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SOME OBSERVATIONS ON ACETAZOLAMIDE-INDUCED NEPHROCALCINOSIS IN THE RAT

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In an article by Harrison and Harrison (1955) it was claimed that nephrocalcinosis could be caused, in young rats fed a high calcium, high phosphate diet, by the administration of the carbonic acid anhydrase inhibitor acetazolamide. If a high calcium, low phosphate diet was used, renal pelvis calculi resulted. The rats were given a single injection of 5000 i.u. calciferol on the first day. It was thought that the factor responsible for preventing the occurrence of calcification in the control group was urinary citrate which is increased by calciferol, and reduced by acetazolamide administration.

Potassium depletion is known to cause reduction of the urinary citrate excretion in man (Fourman and Robinson, 1953) and in the rat (Evans, MacIntyre, Macpherson and Milne, 1956), and it was felt that this fact could be used to provide confirmation of the essential rôle of urinary citrate in this type of experimental nephrocalcinosis. The results obtained in experiments designed to investigate this matter are reported below.

METHODS

Male rats of the M.R.C. hooded strain were used, and the weights are given below, for each experiment. A standard diet was employed throughout—a prepared casein-dextrin-arachis oil mixture with added vitamins, as described by Cooke, Segar, Cheek, Coville and Darrow (1952). This diet is used here as the standard one for potassium depletion. To this were added di-sodium hydrogen phosphate and calcium lactate to give 7 mg. calcium and 5·6 mg. phosphate per g. of food. Acetazolamide ("Diamox", Lederle), where used, was added in a concentration of 2·5 mg. per g. of food.

Experiment I

Three groups of 5 rats were used, weighing between 85 and 110 g. (average, 95 g.).

(*a*) Potassium-depletion group. The animals were potassium-depleted for 1 week by means of a low-potassium diet, ion-exchange resin, sodium-containing drinking fluid and desoxycorticosterone acetate (DOCA) injections. The details have been reported previously

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(Macpherson, 1956). They were then given the high calcium, high phosphate diet (" stock calcium diet "), and to drink, a mixture of 0·25 per cent NaCl and 0·25 per cent $NaHCO_3$ in tap water.

(*b*) Control group. The " stock calcium diet " was given, with a solution of 0·5 per cent KCl in tap water to drink.

(*c*) Diamox group. The animals were given the " stock calcium diet " to which acetazolamide had been added as already described (" Diamox diet "), and 0·5 per cent KCl in tap water to drink.

All the animals were given an injection of 5000 i.u. calciferol (B.P.), subcutaneously, on the first day.

Experiment II

Five groups, each of 6 rats weighing 180–200 g. (average 185 g.) were used.

(*a*) Potassium-depletion group. As for I(*a*).

(*b*) Alkaline Diamox group. As for I (*c*), but the drinking fluid contained 0·5 per cent $NaHCO_3$ as well as 0·5 per cent KCl.

(*c*) Acid Diamox group. As for II(*b*), with 0·5 per cent NH_4Cl in place of the 0·5 per cent $NaHCO_3$.

(*d*) Alkaline control group. As for I (*b*), except that 0·5 per cent $NaHCO_3$ was added to the drinking fluid, as well as 0·5 per cent KCl.

(*e*) Acid control group. As for II (*d*), with 0·5 per cent NH_4Cl in place of the 0·5 per cent $NaHCO_3$.

Each animal was given 5000 i.u. calciferol, subcutaneously, on the first day.

Experiment III

Six groups, each of 6 rats weighing 75–100 g. (average 85 g.) were used.

(*a*) Control, no calciferol. The animals were fed the " stock calcium diet ", and given 0·5 per cent KCl and 0·5 per cent $NaHCO_3$ to drink. They received no calciferol.

(*b*) Control, 2500 i.u. calciferol. As for III (*a*), except that one injection of 2500 i.u. calciferol was given subcutaneously on the first day.

(*c*) Control, 5000 i.u. calciferol. As for III (*a*), except that one injection of 5000 i.u. calciferol was given subcutaneously on the first day.

(*d*) Diamox, no calciferol. As for III (*a*), the " Diamox diet " being used instead of the " stock calcium diet ".

(*e*) Diamox, 5000 i.u. calciferol. As for III (*d*), except that one injection of 5000 i.u. calciferol was given subcutaneously on the first day.

(*f*) Salicylate group. As for III (*e*), but in addition, 0·125 per cent sodium salicylate (B.P.) was added to the drinking fluid.

Urine was collected in metabolism cages over 16-hr. periods, using thymol as preservative. The animals were sacrificed by ether anaesthesia and exsanguination from the inferior vena cava.

Citrate was estimated by the method of Natelson, Lugovoy and Pincus (1947). Capillator pH estimations were checked by glass electrode in doubtful cases. Calcium in blood and urine was estimated by flame photometry (MacIntyre, 1956). Analysis of calcium in kidney was by wet ashing (concentrated nitric acid followed by concentrated perchloric acid) and flame photometry. Chelation of urinary calcium was estimated by passage through an ion-exchange column (sodium sulphonic resin, Bayer), the specimen being sampled before and after each passage through the column. The percentage of " chelated calcium " was taken as the percentage of calcium remaining in the urine after three passages through the column. Estimation and fractionation of urinary salicylate was by the method of Smith, Gleason, Stoll and Ogorzalek (1946).

RESULTS

Towards the end of the third week, the Diamox group in Experiment I were all obviously ill, even moribund, and after sacrificing there was naked-eye evidence of heavy renal calcification. This was confirmed histologically and there was marked tubular dilatation and renal damage (Fig. 1). As in the report by

Harrison and Harrison (1955), most of the calcium was found at the cortico-medullary junction, but there was also a considerable amount in the cortex itself. The control group, however, also showed a very significant degree of calcification both naked-eye and histologically, though there was less evidence of renal damage (Fig. 2). The potassium-depleted group showed no naked-eye nephrocalcinosis though histologically it was still of significant degree (Fig. 3). The mean daily urinary citrate was high in the control group, reduced in the Diamox group, and lowest in the potassium-deficient animals.

These results appeared incompatible with the hypothesis put forward by Harrison and Harrison (1955). A possible alternative, at this stage, was that the mechanism causing the calcification might be analogous to that seen in the human " milk-alkali " syndrome. The next experiment was designed to test this possibility, as well as confirm the findings in the first experiment.

The animals in Experiment II were also sacrificed at three weeks, and the results of the analyses carried out are shown in Table I. The most striking feature is the complete lack of correlation between the degree of renal calcification and any of the other results. The other feature is the way in which the addition of ammonium chloride to the drinking water has almost completely prevented the deposition of calcium, even in the " Acid Diamox " group, even though urinary citrate is practically abolished. There is a considerable difference in kidney calcium content between the " Alkaline Diamox " and " Alkaline control " groups—this appeared to be much greater than the difference between these groups in Experiment I. It was thought that the use of larger animals might account for this. The effect of calciferol in rats of this age-group is very variable, and in any case, since the animals were twice as large, the effective dose would be only half that in the first experiment.

TABLE I.—*Results of Tissue and Urinary Analyses in Experiment II.*

	Kidney calcium mg./100 g. wet kidney. Mean value and range.	Mean daily urinary citrate m. mol./kg. rat/day.	Mean daily urinary pH.	Mean daily urinary calcium mg./kg. rat/day.	Mean daily urinary vol. ml./kg. rat/day.	Mean plasma calcium mg./100 ml.
Potassium-depleted	400 98–974	0·15	7·4	6·5	113	12·2
Alkaline Diamox	5800 3330–9020	0·34	6·6	11·2	137	12·7
Acid Diamox	240 107–353	0·14	6·2	9·7	97	12·9
Alkaline control	90 56–99	0·62	7·6	7·8	57	12·3
Acid control	80 49–93	0·06	6·1	4·6	35	11·9

For explanation of the group names, see text—" Methods."

The third experiment was designed to test the effect of different doses of calciferol, and also to confirm the effectiveness of salicylate in preventing the development of calcification, as reported by Prien and Walker (1956).

The animals were killed after two weeks on the diets, and the kidneys were analysed. Additional estimations were done on a group of stock rats, which had been maintained on the normal cube feed and tap water. These rats were of the

same age and weight as the test animals. Table II shows the amount of calcium present per 100 g. fresh kidney, the mean daily calcium excretion and mean daily urinary volume per kg. rat, and the percentage of " chelated calcium " remaining after the third passage through the ion-exchange column. Fig. 4 shows diagrammatically the relationship between " chelated calcium " and total calcium in the *pooled* specimens of urine tested.

TABLE II.—*Experiment III.—Tissue and Urinary Calcium Analyses.*

	Kidney calcium in mg. per 100 g. fresh kidney.	Mean daily urine calcium in mg. per kg. rat per day.	Mean daily urine vol. in ml. per kg. rat per day.	Percentage of chelated calcium in urine.
Control, no calciferol	48	4·1	56	44
Control + calciferol, 2500 i.u.	68	4·0	59	37
Control + calciferol, 5000 i.u.	52	3·95	36	22
Diamox, no calciferol	105	4·4	61	33
Diamox + calciferol, 5000 i.u.	141	7·45	42	12·5
Diamox + calciferol + salicylate	71	11·25	36	47
Stock rats	30	—	—	—

All animals except the stock rats, received the " stock calcium diet." " Percentage of chelated calcium in urine " = percentage of original calcium remaining in the urine after 3 passages through the ion-exchange column.

It will be noted that while the amounts of calcium found are much smaller than in Experiment II, the kidneys of the rats given acetazolamide contain significantly more than those of the animals in the other groups. The mean daily calcium excretion is also significantly higher. It appears from the Table that calciferol and acetazolamide have a synergic effect which is partially neutralised by giving salicylate, in spite of a rise in the mean daily urinary calcium excretion.

Analysis of pooled aliquots of urine from the salicylate group showed a total salicylate level of 125 mg. per 100 ml., of which 35 per cent was present as free salicylate, 14 per cent as salicyluric acid and 51 per cent as glucuronic acid conjugates.

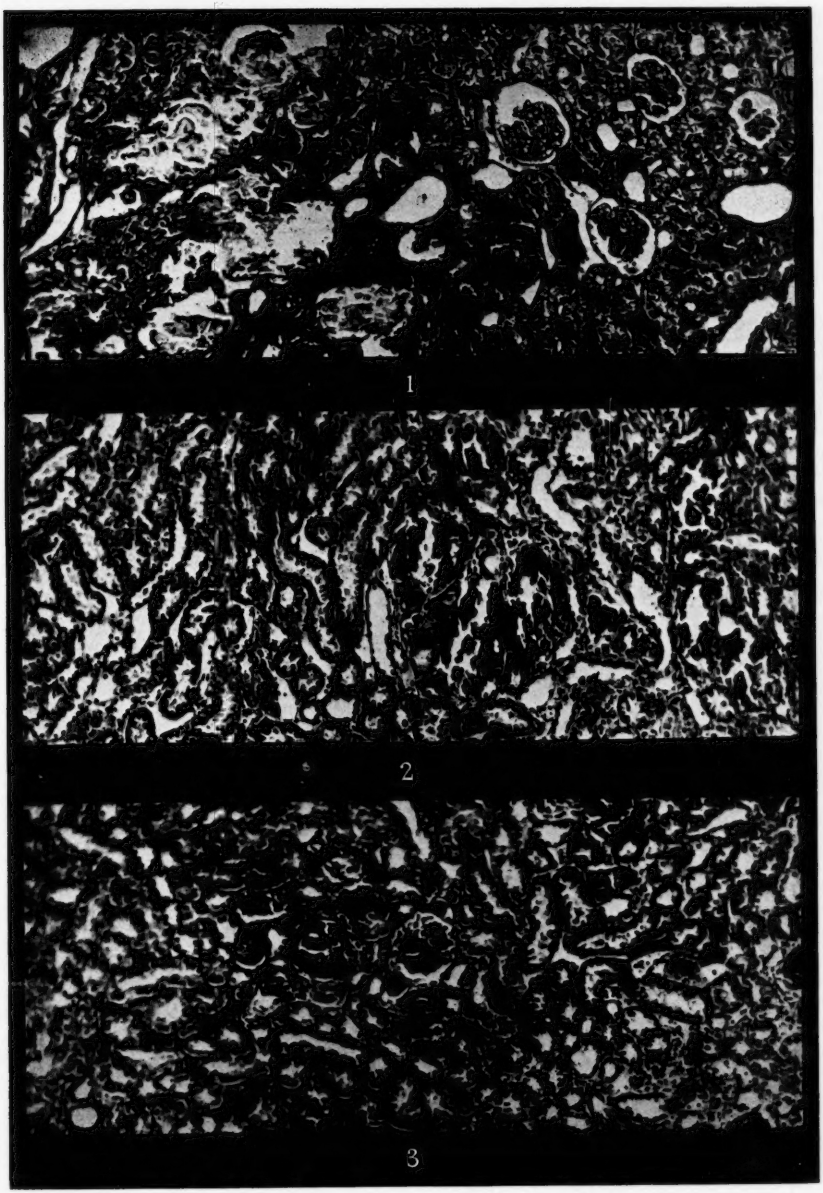
In view of the markedly higher degree of calcification at 3 weeks in Experiment II as compared with that found at 2 weeks in Experiment III, the mean daily calcium excretion in Experiment II was plotted for the "Acid Diamox", "Alkaline Diamox " and " Alkaline control " groups (Fig. 5). There is a marked increase at about the 11th day in both Diamox groups and at about the 16th day in the

EXPLANATION OF PLATE.

FIG. 1.—Kidney of acetazolamide-treated rat (Experiment I). Note the large deposits of calcium and amorphous débris, and the tubular and Bowman's space dilatation. Haematoxylin and eosin. (× 110.)

FIG. 2.—Kidney of control rat (Experiment I). Calcium deposits are smaller than in Fig. 1 and there is less tubular dilatation. Haematoxylin and eosin. (× 110.)

FIG. 3.—Kidney of a potassium-depleted rat (Experiment I). Note the small deposits of calcium and absence of tubular dilatation. Haematoxylin and eosin. (× 110.)



Evans and Macpherson.

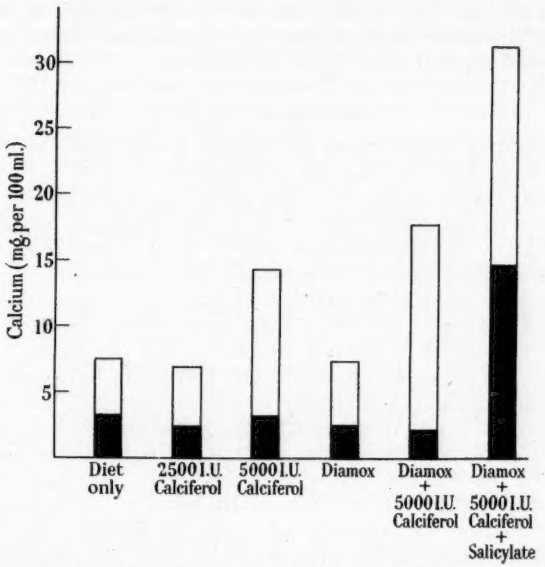


FIG. 4.—Percentage of "chelated calcium" in pooled urines (Experiment III). The height of each block corresponds to the total calcium level in the pooled, untreated specimen of urine. The black portion represents the amount of calcium left after three passages through the ion-exchange column. Results are expressed as mg. per 100 ml. urine in each case.

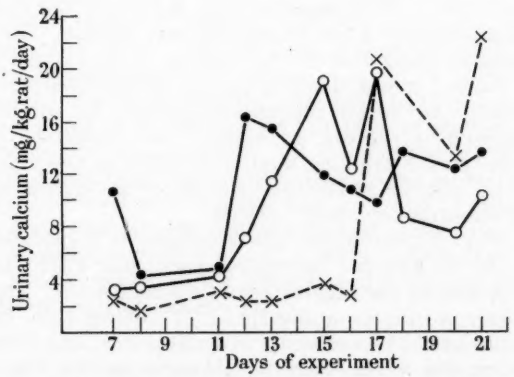


FIG. 5.—Mean daily urinary calcium excretion (Experiment II).

●——● = "Alkaline Diamox" group.
○——○ = "Acid Diamox" group.
× - - - × = "Alkaline control" group.

Note that the excretion is expressed as mg. calcium per kg. rat per day in each case.

alkaline control group. The " Acid control " group showed a similar pattern, though on a slightly lower level, while the " Potassium-depletion " group level remained relatively stable.

DISCUSSION

From the foregoing results there seems little doubt that the calcifying effect of acetazolamide reported by Harrison and Harrison (1955) is important, in the very artificial conditions in which it was tested by them and by us. Equally, there seems little doubt that whatever the mechanism by which this nephrocalcinosis is initiated, urinary citrate is of little or no importance. In an analysis of their data, it appears that in this part of the experiment only 4 test and 2 control animals were used. The amount of calcium in the kidneys of the test group varied from 355 mg. per 100 g. fresh kidney right down to 27 mg. in the fourth animal. This latter figure is a minute amount of calcium (about 0·13 mg. per kidney in this size of rat) which would fall within normal limits by our method of analysis. Their method is obviously less sensitive than ours, since they were unable to estimate the traces of calcium found in the control kidneys, but even so, the range they quote is very wide. The much higher figure for kidney calcium that we report may be due to this difference in methods of analysis, to variations in the régimes, to differing individual susceptibilities, or to a combination of these.

Individual variation undoubtedly plays an important part in determining the degree of calcification, and we feel that this may well be the explanation of the differences between Experiments I and II. The difference between the degree of calcification at 2 and at 3 weeks may be connected with the dramatic rise in urinary calcium in the third week (Fig. 5).

One of the features of acetazolamide is that its effect is self-limiting, disappearing when the plasma bicarbonate level falls below 18 m. eq. per litre (Counihan, Evans and Milne, 1954). If a constant supply of bicarbonate is provided either orally or by infusion, an alkaline urine with high bicarbonate content will continue to be formed. Conversely, administration of an acidifying salt such as ammonium chloride will completely inhibit the action of acetazolamide, provided the plasma bicarbonate is reduced below the critical level of 18 m. eq. per litre. From the results shown in Table I it is difficult to escape the conclusion that in some way the action of carbonic anhydrase inhibitor specifically favours the deposition of calcium ; otherwise it is difficult to explain the protective effect of adding ammonium chloride to the drinking fluid. Alkalinity of the urine cannot be the explanation for the calcium deposition in view of the results in the " Alkaline control " and " Potassium-depletion " groups.

In the same way, hypercalcuria alone cannot be responsible, though it is interesting that the " Diamox " groups in each case have the highest urine calcium output, as well as the highest serum calcium levels. This might suggest that acetazolamide was increasing absorption of calcium from the intestine, but total balance studies would be necessary to confirm this, and to exclude mobilisation of bone calcium due to the acidosis. However, in this connection it will be noted that the " Acid control " group on the same dose of ammonium chloride as the " Acid Diamox " group, have a comparatively normal urinary calcium. Quantitative collection of faeces in rats fed this diet is technically extremely difficult since they have very bulky and semi-solid stools, but it is hoped to investigate this point later.

It has been suggested that urinary colloids are of importance in preventing calculus formation (Butt, 1952), and it was for their similar chelating effect that Prien and Walker (1956) suggested the use of salicylate and salicylamide. The results in Table II suggest that calciferol and acetazolamide reduce the chelating power of the urine, that this effect is additive and that it can be abolished by administration of salicylate. Since calciferol increases the urinary citrate, this would confirm the suggestion that citrate is not the main urinary chelating agent. If the absolute amounts of " chelated calcium " are compared, the reduction is slight (about 25 per cent), though there is a striking reduction in the *percentage*. The increase demonstrated with salicylate administration is impressive by either criterion. We know of no mechanism by which acetazolamide might exert such an effect.

Very recently, Vermeulen, Miller and Chapman (1956) studied the chelating properties of urines from patients forming repeated calculi, and from controls. By ion-exchange column techniques they found only very slight evidence of any chelating activity though there was more evidence of it by dialysis techniques. There was no difference between the patients' urines and the control urines. This apparent discrepancy between their results and ours may be due to at least two factors. Their ion-exchange technique differs considerably from ours, and they were studying humans under relatively normal conditions while we studied rats in grossly abnormal circumstances.

Baker, Reaven and Sawyer (1954), using parathyroid hormone to produce nephrocalcinosis, considered that depolymerisation of the basement membrane polysaccharide was the essential intermediate step. They found that giving DOCA simultaneously prevented this renal calcification, and tentatively suggested that DOCA might increase polymerisation of the polysaccharide. This in turn suggests that the protective effect seen in our " Potassium-depletion " groups is not necessarily due to the depletion, but may be due to the DOCA used in the production of hypokalaemia.

While it is well known that marked nephrocalcinosis can be produced by repeated injections of calciferol, and that this may be evident as early as the 5th day in rats (Pyrah and Raper, 1955) it appears that the single injection used in these experiments had relatively little effect.

We feel that the initiation and perpetuation of stone formation and of nephrocalcinosis is usually due to a complicated interplay of multiple factors, most of them ill understood, and some possibly not yet discovered. For this reason we have no intention of claiming that the nephrocalcinosis produced in these rats is directly related to the level of " chelated calcium " and to total urinary calcium excretion. However, most of the common causal factors can be excluded, the percentage of " chelated calcium " shows a fairly close inverse correlation with the amount of calcium found in the kidney, and salicylate markedly increases chelation, and decreases nephrocalcinosis.

SUMMARY

The observation that nephrocalcinosis can be produced by giving acetazolamide to young rats fed a high calcium, high phosphate diet, is confirmed. No correlation could be found between the degree of nephrocalcinosis and the urinary citrate level, urine calcium, pH or volume. There was moderately good inverse

correlation with the chelating power of the urine, as measured by passage through a simple ion-exchange column.

In these experiments, acetazolamide and calciferol both reduced the percentage of " chelated calcium " in the urine, and this effect was additive. Salicylate, given by mouth, increased the chelating power of the urine and partially prevented nephrocalcinosis in spite of increasing urinary calcium excretion.

It appears possible that acetazolamide increases the absorption of calcium from the intestine.

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IMMUNO-CHEMICAL STUDIES ON BOVINE RIBONUCLEASE

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CRYSTALLISED bovine ribonuclease contains several chromatographic fractions (Martin and Porter, 1951 ; Hirs, Moore and Stein, 1953), of which two have enzyme activity. It is our purpose to examine these fractions by immunological methods and to lay the foundation for further kinetic studies (Cinader, 1955) of the interaction between enzyme, antibody and substrate.

Bovine ribonuclease is one of a small group of protein antigens with a molecular weight lower than 20,000. This low molecular weight restricts its combining capacity with antibody to only a very few molecules of antibody per molecule of antigen. Most of the current hypotheses on antigen-antibody combination have been tested with antigens of a molecular weight between 40,000 and 80,000 (Boyd, 1945 ; Pennell and Huddleson, 1938). With ribonuclease it is possible to test on an antigen of which the molecular weight places it near the boundary between antigen and hapten.

MATERIALS AND METHODS.

The methods employing electrophoresis in agar (Grabar and Williams, 1953 ; Williams and Grabar, 1955*a*, *b*) and agglutination of tanned sensitised erythrocytes (Boyden, 1951 ; Borduas and Grabar, 1953) are described in an earlier paper (Cinader and Dubert, 1955) and details of materials and methods are only given where they differ from those already described.

Protein Antigens

Bovine ribonuclease.—Two preparations of bovine ribonuclease were used. One was a crystallised preparation (Armour, 381–59) and one (89/4) was prepared by Dr. K. Smith.

Fractionation of bovine ribonuclease by chromatography.—The method of Hirs *et al.* (1953) was used. Ribonuclease was freeze-dried after de-salting. Some ribonuclease B fractions were dialysed against distilled water for use in quantitative precipitation experiments.

Analytical Methods

Micro-Kjeldahl.—Proteins were digested with mixtures of H_2SO_4, K_2SO_4, $CuSO_4$. All reagents were M.A.R. grade. The digest was distilled in a Markham (1942) apparatus into 2 per cent (w/v) boric acid containing the indicator described by Ma and Zuazaga (1942).

Enzyme assay of ribonuclease.—Ribonuclease activity in supernatants from antigen/antibody precipitates was measured by a manometric method described by Bain and Rush (1944). The substrate, ribonucleic acid (Nutritional Biochemical Co.), was purified by the method of Kunitz (1940). The side arms of the Warburg flasks contained 0·1 ml. A blank was included in every test, the side arm containing the same concentrations of serum and buffer as the supernatant fluids. The activity of the supernatants was corrected for the CO_2 evolved in this blank. The activity was recorded as the corrected volume of CO_2 evolved between the 10th and 60th minute after the addition of enzyme to substrate.

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Immunological Methods

Immunisation of rabbits.—All anti-sera were obtained from rabbits initially immunised by Freund and McDermott's method (1942) and later by intravenous injections with crystalline ribonuclease at intervals of 1–2 months.

Diffusion of antigen against antibody in agar gel (Ouchterlony, 1949 ; Oudin, 1952).—Washed and filtered agar was made up in 0·15 M-NaCl (0·75 per cent agar) with 1/10,000 merthiolate. The agar was poured into petri dishes or into containers consisting of a bottom glass plate and a square perspex plate in which a circular hole was cut. The perspex plate was attached to the glass plate by transparent tape. Cylindrical holes were cut in the agar and the bottom of each hole was covered with 2 drops of agar to form a cup, which was filled with the reagent. Petri dishes were sealed with plasticine. The perspex plate was covered with a flat glass plate and the perspex glass seal was made water-tight with tape.

Agglutination of tanned and sensitised red cells.—The concentration of the cell suspension was adjusted so that when 0·5 ml. of suspension was added to 7·5 ml. of distilled water and treated with coal gas for 5 min. the optical density of the resulting solution was 0·3 (EEL Colorimeter, Ilford filter 624, colorimeter tubes of 1·5 cm. diameter). After sensitisation and washing of the cells, their concentration was again adjusted to give the same optical density when lysed. To each serum dilution, 0·05 ml. of cell suspension was added.

Inhibition of agglutination.—The antigens were examined for ability to inhibit the agglutination of tanned and sensitised red cells by anti-serum previously heated at 56° for 1 hr. and absorbed with red cells. The agglutination titre of mixtures was determined and compared with that of anti-serum.

Removal of complement from sera used in nitrogen assay of antigen-antibody precipitates (Heidelberger and Anderson, 1944 ; Maurer and Talmage, 1953). Complement in ribonuclease anti-sera was reduced by treatment with precipitates prepared from human albumin and rabbit anti-sera to human albumin. In these precipitates the antigen and antibody were in equivalent proportions. Samples containing 100 μg. N were added to each ml. of undiluted ribonuclease anti-serum. After 2 days at 1° ± 1° the precipitates were separated from the serum by centrifuging.

Nitrogen assays of antigen/antibody precipitates.—Varying amounts of antigen were added to constant quantities of antibody and immediately mixed to reduce the Danysz effect (Danysz, 1902). Mixtures were adjusted to constant volume with phosphate saline buffer (pH 7·5 ; 0·0062 M-Na_2HPO_4, 0·00135 M-KH_2PO_4, 0·15 M-NaCl). Antigen was added with an " Agla " syringe. The mixtures were kept at 37° for 2 hr. and then at 2° for 6 days. The protein precipitated was centrifuged at 4°, washed twice with 2 ml. of ice-cold 0·15 M-NaCl and then assayed for nitrogen by the micro-Kjeldahl method. Mixtures of antigen and saline buffer and mixtures of anti-serum and saline buffer gave no significant N values when subjected to the same incubation, isolation and digestion procedures.

RESULTS

Diffusion and Precipitation in Agar Gels

The chromatographic fractionation of crystallised bovine ribonuclease is shown in Fig. 1. The numbers in the Figure refer to the contents of individual tubes. We have compared the contents of individual tubes and not pooled fractions except with ribonuclease A when the contents of the tubes were pooled and freeze-dried.

A comparison of fractions by diffusion and precipitation in agar (Fig. 2) showed that all the chromatographic fractions had an antigen in common, shown by the common zone (*cf.*, Crumpton and Davies, 1956). A second zone appeared in the fractions (tubes 1–14) first eluted from the chromatographic column ; the substance responsible for this second zone of precipitation was not present in ribonuclease A or B. After prolonged development the zone of this common antigen broadened out and in its course across the agar, in relation to the cups containing the different chromatographic fractions, seemed to twist and turn

(Fig. 2, development : 7 days). A fine structure of periodically spaced lines could usually be seen within these bands. Although the continuity of the band indicated immunological identity, it was conceivable that the twisting of the precipitin zones was caused by the presence of two or more antigens in each chromatographic fraction and that the fractions differed from one another in the relative concentration of these components. However, a similar phenomenon was produced by allowing the antibody to react with graded concentrations of ribonuclease A (Fig. 3). A precipitate was initially deposited in a narrow zone and became the stationary boundary of a zone broadening towards the cup containing

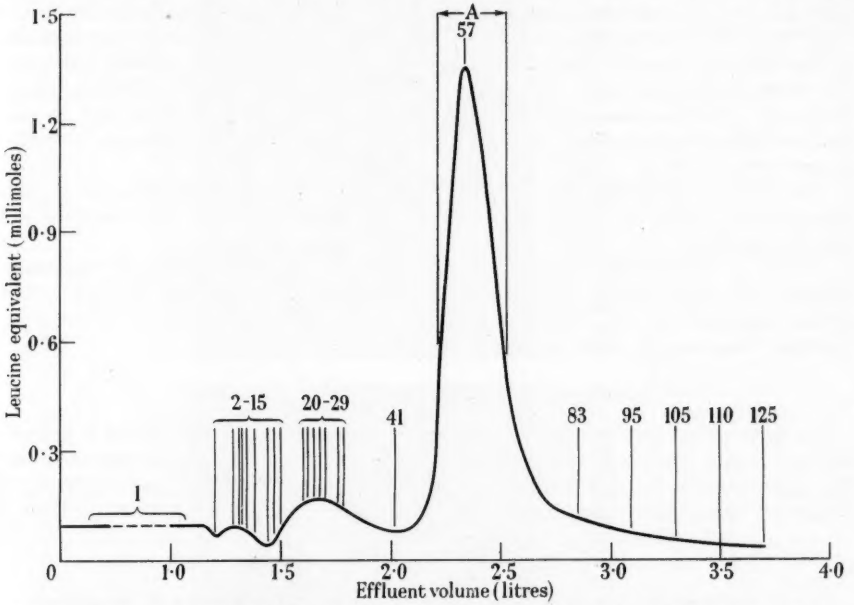

FIG. 1.—Chromatographic fractionation of bovine ribonuclease (Armour 381—59) by the method of Hirs, Moore and Stein (1953). Numbers refer to the contents of individual tubes used in later experiments.

the antibody, if antigen was in excess, or towards the cup containing the antigen, if antibody was in excess (Cinader, Rondle and Pearce, 1955). The broadening of the zone could thus be attributed to an anomaly in the interaction of a single antigen with its antibody. It is conceivable that a zone due to an antigenic impurity could have been hidden by the broad precipitation zone of the enzyme. To reduce this risk, diffusion in agar gel was carried out with supernatants from antigen-antibody precipitates of ribonuclease A and B. No evidence for an antigenic impurity was found (Fig. 4).

The agar gel tests showed, therefore, that there was a common antigen in all the fractions ; that the leading chromatographic fractions (Fig. 1) contained a second antigen, and that ribonuclease A and B were not contaminated with this second antigen. There were no differences between ribonuclease A and B.

Electrophoresis in Agar

Prolonged electrophoresis (16 hr., 10 mA) in agar (2 per cent w/v, glycine buffer, pH 8·4 ; $I = 0{\cdot}05$) followed by diffusion of ribonuclease anti-serum from lateral channels, showed that both ribonuclease A and B migrated as two components. The zone of precipitation was continuous, but had two maxima (Fig. 5a). Immediately after electrophoresis in agar, portions of the agar were cut out in positions corresponding to the predetermined areas of highest antigen concentration, inserted into fresh agar slides and subjected to electrophoresis. At the end of this second run anti-serum was allowed to diffuse from the median channels ; each component now migrated as a single electrophoretic component (Fig. 5b). Thus the electrophoretic differences appeared to be due to heterogeneity of the starting material and not to a change during the course of electrophoresis. We were unable to show this electrophoretic heterogeneity by electrophoresis on paper. We concluded that our preparations of ribonuclease A and B were antigenically homogeneous but contained two components of different mobility in agar gels.

We have occasionally observed twinning of the antigen-antibody zones in these experiments ; that is, the formation of two zones, close to one another and exactly parallel.

In one of the ribonuclease preparations, 89/4, the concentration of the second antigen was high enough to be demonstrable by electrophoresis in agar. This second antigen moved with the same mobility as the slower of the two ribonuclease components (Fig. 5c).

Agglutination and Inhibition of Agglutination

It is possible to distinguish between human albumin and diazo *p*-benzene sulphonic acid human albumin by the degree to which the two antigens inhibit the agglutination by anti-serum to diazo human albumin of tanned erythrocytes sensitised with diazo human albumin (Cinader and Dubert, 1956). Human

EXPLANATION OF PLATES.

FIG. 2.—Diffusion and precipitation in agar of chromatographic fractions of ribonuclease (Armour) and ribonuclease anti-serum (from rabbits immunised with Armour ribonuclease). t, Tube numbers (Fig. 1) ; S, Ribonuclease anti-serum.

FIG. 3.—Diffusion and precipitation of graded quantities of ribonuclease A (t 57) against ribonuclease anti-serum. Development : 14 days. Arrows indicate the position of the narrow zones laid down initially.

FIG. 4.—Diffusion and precipitation in agar of supernatants from a quantitative precipitin experiment (Fig. 10, serum 570, ribonuclease A) ; l.h.s. diffusion and precipitation of supernatants against ribonuclease A (centre row) ; r.h.s. diffusion and precipitation against ribonuclease anti-serum (centre row) ; —→ Equivalence zone.

FIG. 5.—Electrophoresis in agar of ribonuclease and fractions of ribonuclease, followed by diffusion of ribonuclease anti-serum from a median channel. The left-hand side of each of the agar slides was connected to the + ve electrode.

FIG. 5a.—Electrophoresis in agar of ribonuclease A and B (Rn A, Rn B). The median channel contained ribonuclease anti-serum.

FIG. 5b.—Electrophoresis in agar of fractions of ribonuclease A and B cut out of an agar slide after an initial electrophoresis experiment (Rn 1B, Rn 2B, Rn 1A, Rn 2A). The median channel contained ribonuclease anti-serum.

FIG. 5c.—Electrophoresis in agar of bovine ribonuclease (Rn Armour 381—59 and Rn 89/4). The median channel, containing the anti-serum, was at the top of each of the two photographs shown.

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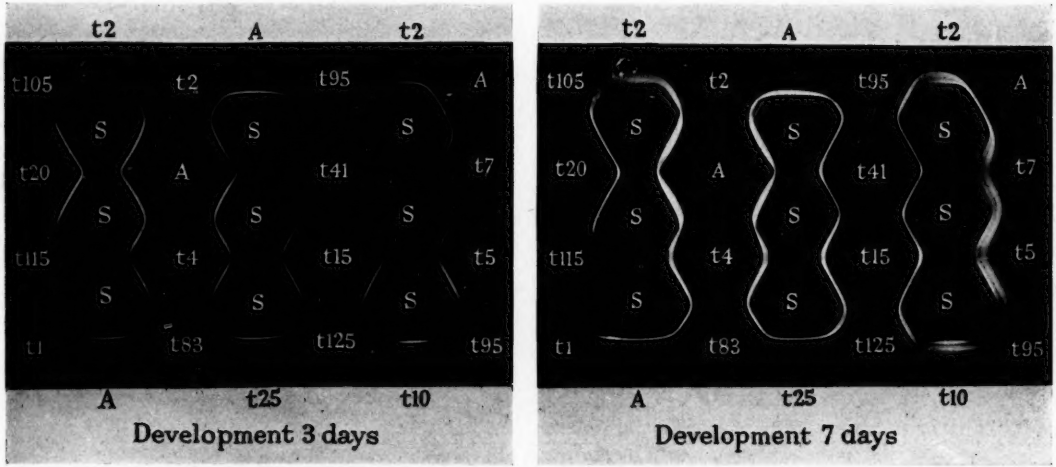
t2
A
t2
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t2
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S
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t4
t15
t5
S
S
S
t1
t83
t125
t95
A
t25
t10
Development 3 days
t2
A
t2
t105
t2
t95
A
S
S
S
t20
A
t41
t7
S
S
S
t115
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Development 7 days

Fig. 2.

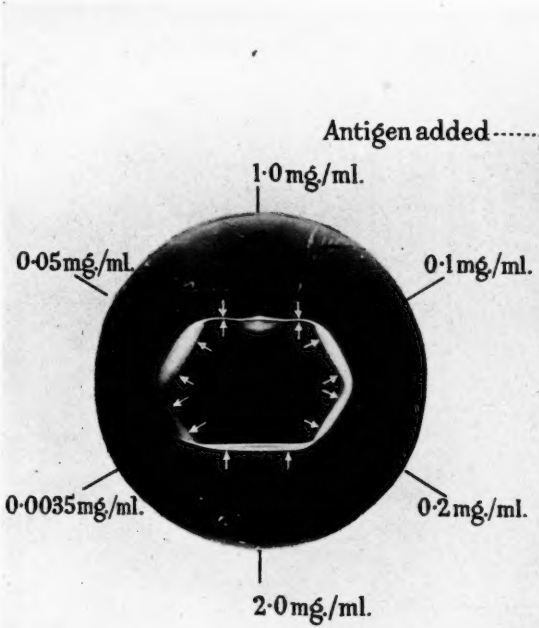

Fig. 3.

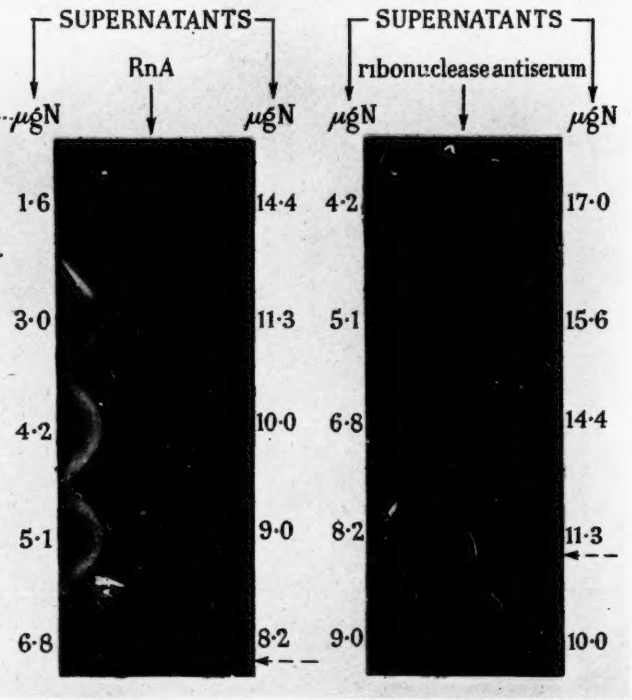

Fig. 4.

Cinader and Pearce.

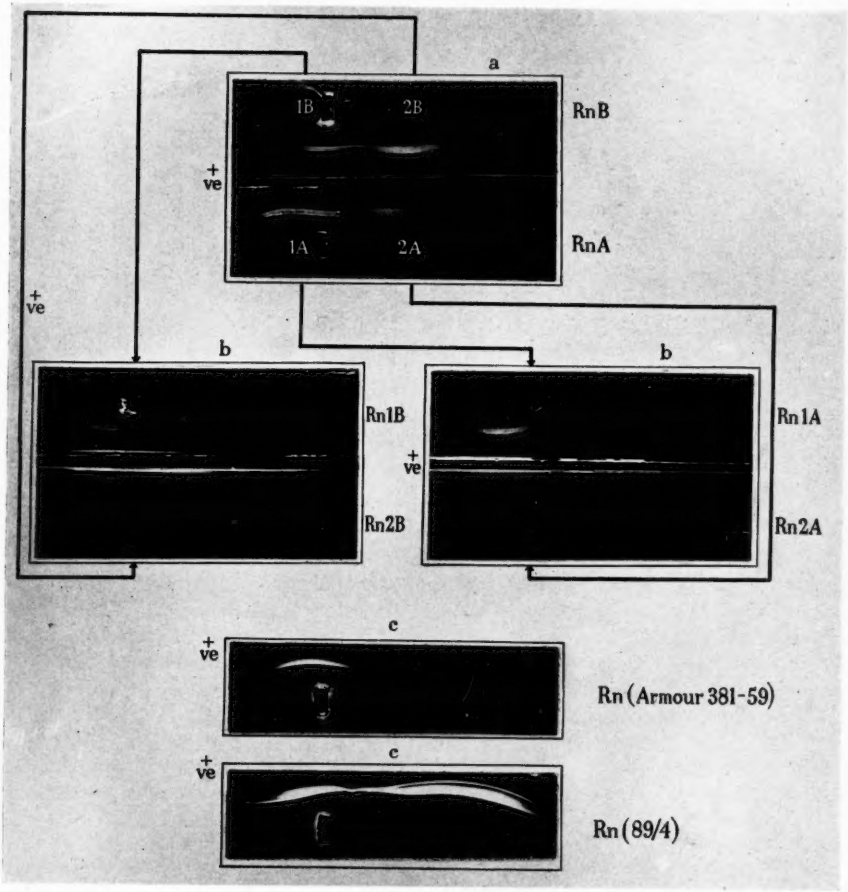
a
1B
2B
RnB
+ ve
1A
2A
RnA
+ ve
b
b
Rn1B
Rn2B
Rn1A
+ ve
Rn2A
c
+ ve
Rn (Armour 381-59)
c
+ ve
Rn (89/4)

Fig. 5

albumin inhibits this agglutination partially, whereas diazo human albumin inhibits it completely. This method also reveals gross differences in combining power. Two ribonuclease preparations, of which one, 89/4, was known to contain considerable quantities of the second antigen, were compared by precipitation and inhibition of agglutination. The difference in inhibitory power as shown by inhibition of agglutination agreed with that observed by quantitative precipitin tests (Fig. 6).

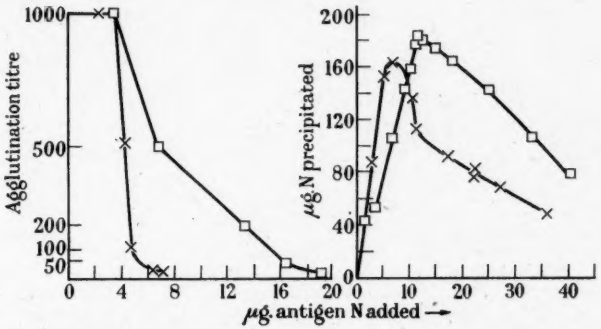

FIG. 6.—A comparison of two ribonuclease preparations (Armour and 89/4) by inhibition of agglutination and by quantitative precipitin tests.

Erythrocytes sensitised with Armour ribonuclease.
× Armour ribonuclease.
□ Ribonuclease 89/4.

A difference between antigen specificity or combining power between ribonuclease A and B might be revealed by inhibition of agglutination.

Ribonuclease A and B were compared by sensitising tanned erythrocytes with ribonuclease A, ribonuclease B and crystallised ribonuclease respectively and inhibiting the agglutination of sensitised cells with each of these three antigens. There was no difference between ribonuclease A and B (Fig. 7). The agglutination was also inhibited completely by fractions containing the second antigen, but slightly more antigen nitrogen was required to achieve this.

Inhibition tests thus failed to distinguish between ribonuclease A and B. This method can only give information in the zone of antibody excess and it was necessary to examine quantitative precipitin curves for ribonuclease A and B to obtain information in the zone of antigen excess. A detailed examination of the quantitative precipitin curve of ribonuclease A served as a basis for this comparison.

Assay of Nitrogen in the Precipitates of Ribonuclease A.

The nitrogen content of precipitates formed by ribonuclease A with rabbit anti-serum (619) is shown in Table I. The limits of the equivalence zone were determined by flocculation, by agglutination of tanned erythrocytes sensitised with ribonuclease A, by enzyme assays of the supernatant and by diffusion and precipitation in agar of the supernatants against antigen and antibody respectively. The co-existence of antigen and antibody in the same supernatants could not be shown by any of these tests.

The precipitates were not completely soluble in antigen excess ; the precipitin curve tailed off in this region. The composition of the antigen-antibody precipitate in the region of partial solubility was determined by a procedure similar

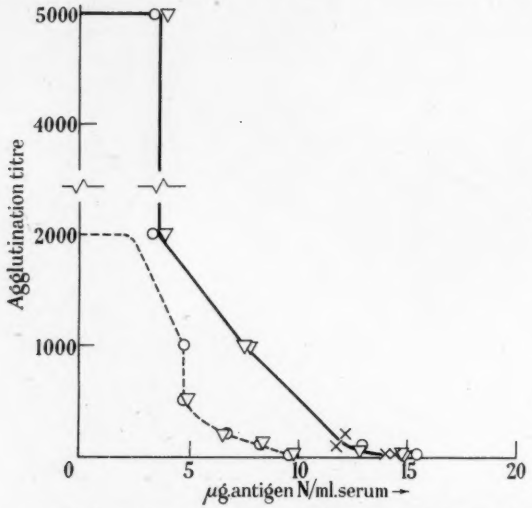

FIG. 7.— A comparison of ribonuclease A and ribonuclease B by inhibition of agglutination.
——— Serum Pool 1, tanned erythrocytes sensitised with crystallised ribonuclease.
--------- Serum Pool 2, tanned erythrocytes sensitised with ribonuclease A.
Antigen used for inhibition of agglutination :
○ Ribonuclease A.
▽ Ribonuclease B (t 24).
× Crystallised ribonuclease.

TABLE I.—*Precipitation of Ribonuclease by Rabbit Antibody*

Addition of increasing amounts of ribonuclease to 0·05 ml. of serum 619.

Antigen N (μg.).	Total N precipitated (μg.).	Ratio antibody N : to antigen N in precipitate.	Molecular ratio (antibody to antigen) in : Precipitate.	Molecular ratio (antibody to antigen) in : Supernatant.	Tests on supernatant. Antigen. Flocculation.	Antigen. Enzyme activity as μg. N Rn A/3 ml.	Antibody. Flocculation.	Antibody. Agglutination titre.
1·0	34·4	33·4	2·8	..	—	—	+	
2·0	66·8	32·6	2·73	..	—	—	+	200
3·05	101·0	32·1	2·69	..	—	—	+	200
3·95	118·9	29·1	2·44	..	—	—	+	200
4·9	135·2	26·4	2·21	..	—	—	+	50
5·9	148·2	24·0	2·01	..	—	—	—	50
7·4	177·6	22·9	1·92	..	—	—	—	10
8·9	185·7	19·9	1·67	..	—	—	—	1
9·8	191·4	18·5	1·55	..	—	—	—	<1
10·2	188·4	17·7	1·48	..	+	0·5	—	<1
13·2	138·5	15·9	1·33	0·85	+	3·8	—	<1
15·3	106·4	16·7	1·40	0·73	+	7·2	—	<1
17·3	83·5	17·8	1·49	0·70	+	10·2	—	<1
19·3	69·2	17·0	1·42	0·64	+	13·6	—	<1

to that used by Heidelberger and Kendall (1935*b*). The stoichiometric composition of the precipitates was calculated, assuming a molecular weight of 13,400 for ribonuclease A, of 160,000 for rabbit antibody (Tiselius and Kabat, 1939; Nichol and Deutsch, 1948) and identical N content of ribonuclease and antibody. For rabbit antibody the molecular ratio of antibody to antigen was 2·8 in the antibody excess zone, and 1·45 in the antigen excess zone (Table II); the ratio in the equivalence zone was between 2·0 and 1·55 when the position of the equiva-

TABLE II.—*Molecular Ratio (Rabbit Antibody/Ribonuclease A) in Region of Antigen Excess Serum* 619

Rn N added to 0·16 ml. of serum (μg.).	Total N ppt. (μg.).	Specific N in supernatant (μg.).	Vol. anti-serum added to supernatant (ml.).	Total N ppt.* in analysis of supernatant (μg.).	Antibody N precipitated (from serum added to supernatant) (μg.).	Per cent Rn N in 2nd ppt.	Rn N in entire supernatant (μg.).	Rn N in ppt. (μg.).	Molecular ratio of antibody to antigen in ppt.
32·6	602·7	11·0	0·028	9·0	1·7	2·2	0·4	32·3	(1·48)
42·4	443·1	180·4	0·12	350·2	229·9	3·1	19·3	26·3	1·33
48·9	340·5	289·5	0·188	594·6	401·5	3·4	30·65	18·3	1·40
55·4	267·2	369·3	0·228	796·6	550·4	3·45	41·2	14·2	1·49
61·9	221·5	421·5	0·252	936·7	655·7	3·5	49·6	12·3	1·42

* All values determined on ¾ of supernatant.

lence zones was determined by flocculation and 1·55 when determined by agglutination of sensitised and tanned cells and by enzyme assays of the supernatant.

Data covering the range of antibody excess to equivalence were substituted in the equations of Heildelberger and Kendall (1935*a*), Ghosh (1935*a*), Langmuir (1918) and Freundlich (1923); the constants of these equations were computed and the theoretical quantity of precipitating antibody was calculated from these constants and from the amount of antigen added to the serum. The differences between the calculated and the observed values were found and the squares of these differences were summed for each equation. The values calculated from the equations of Heidelberger and Kendall gave the best agreement with experimental values (Table III). The results were in reasonable agreement with the theory advanced by Goldberg (1952) over the whole range examined (Fig. 8).

TABLE III.—*Comparison of Experimental Values (Ribonuclease A-Rabbit Antibody) with Values from Equations Describing the Composition of Immune Precipitates in the Antibody Excess Zone*

Equation.	Authors.	Constants. *a.*	*b.*	*A.*	*B.*	σ^2.
$R = a - bG$	Heidelberger and Kendall (1935*a*)	36·06	1·83	—	—	5·6
$R = a - bG^{\frac{1}{2}}$	" " " "	42·57	7·28	—	—	11·5
$R = ApB$	Freundlich (1923)	—	—	15·3	0·15	16·2
$R = \frac{Ap}{1+Bp}$	Ghosh (1935), Langmuir (1918)	—	—	11·16	0·37	57·7

R = N ratio of antibody/antigen in the precipitate.
G = μg. N antigen added to 0·05 ml. of serum 619, p = concentration of unprecipitated antibody (μg.).
σ^2 = sum of squares of the difference between calculated and observed values.

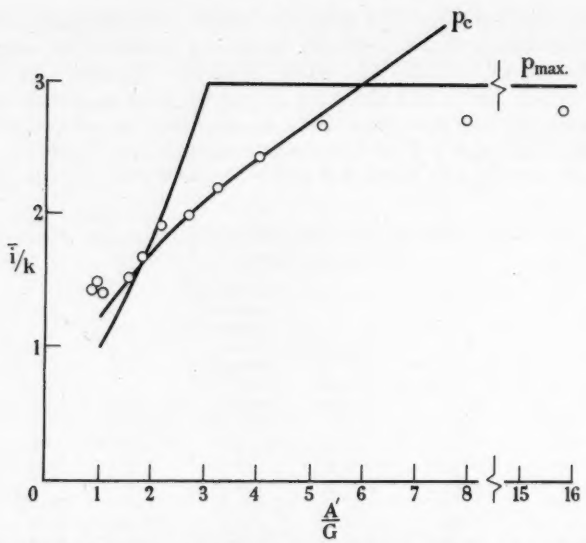

FIG. 8.—The Goldberg Hypothesis. Plot of the limiting values ($P_{max.}$ P_c) computed from Goldberg's hypothesis (1952) assuming the effective valency (f) of the antigen to be 4 and adjusting the antibody/ml. of serum from 3·63 to 3·77 mg. N.

$\bar{i}/k$ = average antibody/antigen ratio of all aggregates containing k antigen molecules.
A'/G = antibody/antigen ratio of the entire system.
o ... values obtained from nitrogen assays of the precipitate formed by ribonuclease A and ribonuclease rabbit anti-serum.

The general nature of these observations was confirmed with other sera. In no case could the co-existence of antigen and antibody in the equivalence zone be shown by flocculation or diffusion and precipitation in agar, but agglutinating antibody and enzyme could be demonstrated in the same supernatants in one instance (Fig. 9).

Precipitin Curves for Ribonuclease B.

Our preparation of ribonuclease B had only 75 per cent of the enzyme activity of ribonuclease A as judged by manometric tests. The precipitin curve of mixtures of ribonuclease B and rabbit antibody does not differ significantly from that of ribonuclease A in the zone of antibody excess (Table IV). Examination of supernatant fluids revealed no antigenic impurity. In antigen excess some sera indicated a complete identity of ribonuclease A and B whereas others indicated a difference, but only in that slightly less nitrogen was precipitated by ribonuclease B in the initial part of the zone of antigen excess (Fig. 9, 10); this difference was never more than 10 per cent of total nitrogen precipitated. The activity of the supernatants of ribonuclease A and B differed in the same manner as that of the two enzyme preparations. There was no evidence of denaturation of antigen by enhanced specific precipitation in the antigen excess zone (Kabat and Mayer, 1948). Dialysis of the preparation resulted in loss of ribonuclease and might be expected to increase the relative concentration of

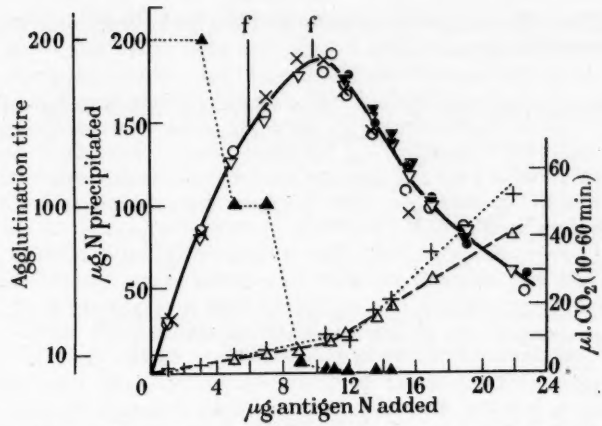

FIG. 9.—A comparison of ribonuclease A and ribonuclease B (t 20, 21, 28, 29) by quantitative precipitin tests.

Ribonuclease anti-serum : pool 4.

f Equivalence zone determined by flocculation test.
× Ribonuclease Armour (381—59).
○ Ribonuclease A (6 days' incubation).
● Ribonuclease A (14 days' incubation).
▽ Ribonuclease B (6 days' incubation).
▼ Ribonuclease B (14 days' incubation).
..+.. Enzyme activity of supernatants.
..▲.. Agglutination titre of supernatant.
—△— Enzyme activity of supernatants.

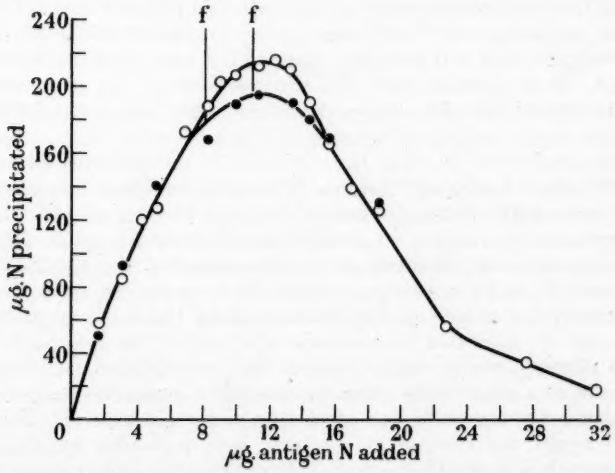

FIG. 10.—A comparison of ribonuclease A and ribonuclease B (t 20, 21, 28, 29) by quantitative precipitin tests.

Ribonuclease anti-serum : 570.

f Equivalence zone determined by flocculation test.
○ Ribonuclease A (6 days' incubation).
● Ribonuclease B (6 days' incubation).

protein impurities of a molecular weight greater than 13.000. Dialysis did not produce any changes.

TABLE IV.—*Comparison of the Precipitin Curves of Ribonuclease A and B in Antibody Excess*

Ribonuclease anti-serum.	*b.* Ribonuclease A.	*b.* Ribonuclease B.	*t.*	$> P >$
Pool 2	1·8	2·0	0·338	0·8 0·7
Pool 3	0·9	1·0	0·351	0·8 0·7
570	1·4	1·6	1·074	0·4 0·3

The value b is determined for the equation $R = a - bG$ (Heidelberger and Kendall, 1935*a*) by computing the best straight line for a plot of R against G (see Table III).
The slopes of these lines (b) for ribonuclease A and B are compared.

DISCUSSION

Crystallised ribonuclease appears to contain two unrelated antigens, ribonuclease and another antigen which is present in the initial chromatographic fractions. Whereas much of the evidence indicates that ribonuclease A and B contain only one antigen, some observations make this conclusion questionable. Three of these may have the same cause : the broadening of the boundary during diffusion and precipitation in agar ; the occasional twinning of the precipitation zone following electrophoresis in agar, and the incomplete solubility of the antigen-antibody precipitate in excess antigen. The first two of these observations may depend on the third. If the antigen-antibody precipitate is only incompletely soluble in excess antigen, an antigen-antibody zone once produced would not be completely dissolved by antigen, and if it is partly dissolved, a zone showing striations could perhaps result. It is possible that this process occurs with a number of other antigens (Wilson and Pringle, 1954 ; Salvinien and Kaminski, 1955 ; *cf.* also Oudin, 1955).

During electrophoresis in agar the diffusion of antigen progresses very far before the diffusion of antibody begins. This might reduce the movement of a zone and hence its width, leaving a double boundary which we describe as twinning.

If this argument is valid, a single fact need be considered in discussing the antigenic composition of ribonuclease A—the shape of the precipitin curve in antigen excess. It is by no means certain that deviation from the parabolic shape of the precipitin curve, usually characterising the interaction of a protein and rabbit antibody, indicates the presence of an impurity. It is possible that the shape of the nitrogen assay curve could be the outcome of two superimposed curves, one due to a major, the other to a minor component (Cohn, 1952). We have no evidence for the existence of such a minor component. The precipitin curve for lysozyme, an antigen of similarly low molecular weight, shows the same behaviour (Gonçalves, Molinari and Deutsch, 1956). The tailing of the precipitin curve may prove to be a feature of the antigen-antibody interaction of this class of antigens.

A fourth experimental observation may indicate an antigenic impurity in ribonuclease A and B—the co-existence in supernatants from some sera of agglutinating antibody and of enzyme activity. It is however possible that this

fact could be due to the presence of a soluble antigen-antibody complex. On this assumption molecular ratios were determined from a serum which showed by all methods an equivalence zone free of antibody and enzyme activity.

The nitrogen ratios of antigen-antibody precipitates which we have obtained are not in agreement with those reported by Smolens and Sevag (1942–43). An apparent agreement with values computed by Marrack (1954) from the data of Smolens and Sevag is due to the fact that Marrack has used 17,000 for the molecular weight of ribonuclease whereas we have chosen a value of 13,400 (Fankuchen, 1941 : Carlisle and Scouloudi, 1951 ; Anfinsen, Flavin and Farnsworth, 1952 ; Anfinsen, Redfield, Choate, Page and Carroll, 1954 ; Hirs, Moore and Stein, 1956). The difference between our results and those of Smolens and Sevag (1942–43) could be due to the presence in their preparation of a large quantity of the antigen unrelated to ribonuclease, which we have reported in this paper.

We have failed to detect a difference between ribonuclease A and B by all methods other than the quantitative precipitin method, where a small difference in total nitrogen precipitated was observed with some, but not in all sera.

The heterogeneity of ribonuclease A and B shown by electrophoresis in agar is not an antigenic heterogeneity. It corresponds to a heterogeneity in the crystalline starting material. We can only conclude that chromatographic fractionation does not affect this heterogeneity when it occurs in the crystallised starting material.

We have given our reasons for thinking that ribonuclease A is a single antigen. This conclusion reinforces the arguments presented in the introduction for choosing ribonuclease A in testing theories of antigen-antibody interaction. Of the equations which apply to the antibody excess zone only, those of Heidelberger and Kendall (1935*a*) give the best fit. Our results are compatible with the theory advanced by Goldberg (1952).

SUMMARY

Ribonuclease A and B could not be distinguished by any of the immunological methods used in this paper ; a slight difference in antigen excess was found with some but not all sera.

Crystallised ribonuclease contains in addition to ribonuclease one unrelated antigen which is separated in the first fraction eluted from an amberlite column.

Both ribonuclease A and B were shown to migrate in agar gel with two mobilities. The faster component of A has the same mobility as the faster component of B and the slower component of A has the same mobility as the slower component of B. The antigen unrelated to ribonuclease has the same mobility as the slower ribonuclease component.

Molecular ratios were determined for rabbit antibody and ribonuclease. They were 2·8 in antibody excess ; 1·55 at equivalence and 1·45 in antigen excess.

The quantitative precipitin results were found to be compatible with the theory of Goldberg.

Supernatants of ribonuclease A and B were examined by diffusion and precipitation in agar. No evidence for an antigenic contaminant was found.

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AN ANALYSIS OF CERTAIN CHEMICAL CONSTITUENTS OF RABBIT BONE FOLLOWING DEPOSITION OF ^{90}Sr*

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IT is known that radioactive strontium is retained in the skeleton in high concentration (Kidman, Tutt and Vaughan, 1950) and that it is " par excellence a producer of bone tumours " (Brues, 1949). Little is known, however, about the dose levels that will produce either radiation dysplasia or radiation neoplasia, nor of the chemical and histological changes that may be produced in bone by different dose levels. A long-term experiment has therefore been undertaken to determine (i) at what dose levels bone injury will develop in the rabbit, (ii) the nature of radiation dysplasia. In addition to autoradiographic, microradiographic and histological studies of the bones, comparison between certain chemical constituents in irradiated and normal rabbits is in progress.

Rabbits receiving the highest doses in each age group studied have already died 6–8 months after injection. The following paper reports the results of such injections on the length and weight of the femur and its chemical constitution and must be regarded as part of the study of radiation dysplasia. The processes involved in radiation dysplasia are extremely complex but factual information about differences between normal and irradiated bone, it is hoped, will bring about a better understanding of the mechanisms concerned. The present results of the chemical analysis for instance indicate that the same injected dose affects the constitution of the bone to a greater extent in the adolescent rabbit than in the young or adult rabbit, a difference which can perhaps be explained in physiological terms but clearly indicates that the adolescent is more likely to be affected by the radioactive strontium constituent of " fall-out " than either the baby or the adult.

Estimations of the following bone constituents were made—(i) calcium as a measure of the inorganic content ; (ii) total nitrogen as a rough measure of the organic content ; (iii) hexosamine as an index of mucopolysaccharide content ; (iv) hydroxyproline as an index of collagen content.

METHODS

Rabbits were of the same stock and kept on the same diet as those used in previous experiments (Kidman *et al.*, 1950 ; Jowsey, Rayner, Tutt and Vaughan, 1953). Single intravenous injections of $^{90}SrCl_2$ in carrier-free solution were given to 4 weanling rabbits 5–7 weeks old (500–630 μc./kg.). They died about 6 months later. Similar injections were given to 2 rabbits 48 hr. after birth (500 μc./kg. body wt.). These were killed about 7 months later. Single intravenous injections of ^{90}Sr were given to 4 rabbits aged 1 year (1000 μc./kg.), they also died approximately 6 months later. The cause of death was anaemia and leucopenia. One femur from each rabbit was used for chemical analysis. Femurs from uninjected rabbits

* This work was begun on behalf of the Medical Research Council's Committee on Protection against Ionising Radiations.

of the same ages and where possible litter mates were used as controls. Only one control killed at exactly the same age was available for the rabbits injected at 48 hr. old. The control rabbits for the group injected 6–8 weeks old, however, were only a few weeks older and showed comparable values for weight and length of the femur.

Preparation of bones

The bones were scraped free from muscle and periosteum with a scalpel and then the ends sawn off just below the epiphyseal plate so that the plate was included with the ends. The marrow was washed out with a fine jet of water and the bones then wiped with a piece of wet cotton wool, followed by another wash. The two ends from each femur were pooled. The bones were reduced to powder by cutting on a milling machine (Jowsey, 1955) using a H.S.S. screw slitting cutter (Buck and Hickman Ltd.) $\frac{1}{10}$ in. (0·25 cm.) in thickness, the saw being set so that only powder was produced. The radioactive dust was kept from the atmosphere by cutting under distilled water, the whole sawing table being covered with a perspex case. The machine was washed down when each portion of bone was finished. The powder was filtered off at the pump and dried over a boiling water bath. Lipids were removed by extraction with ethyl ether for 12 hr. in a Soxhlet apparatus. The dried specimens were stored over calcium chloride.

Calcium estimations.—Weighed amounts of bone powder were dissolved in 50 per cent (v/v) nitric acid, evaporated to dryness and ashed in a furnace (400°). Calcium was estimated by the standard oxalate-potassium permanganate method. The oxalate precipitate was filtered off through a × 4 sintered glass Gooch crucible. The experimental error in our hands was ± 1 per cent.

Total nitrogen estimations.—These were carried out by a modification of the micro-Kjeldahl technique, using sodium selenate and mercuric sulphate as catalyst for the digestion of the bone which lasted 2 hr. (Eastoe and Eastoe, 1954). The experimental error was ± 2 per cent.

Hydrolysis of bone powder.—(*a*) For hexosamine analyses. Approximately 0·1 g. bone powder was sealed off in hard glass test tubes with 3 ml. 4N-HCl and heated in a boiling water bath for 4 hr. The ampoules were opened and 1 ml. of the solution removed to other test tubes which were then sealed off. The remaining hydrolysate was neutralised with 2N-NaOH using phenolphthalein as indicator. The volume was made up to 10 ml. and the precipitate filtered off.

(*b*) For hydroxyproline analyses. The ampoules containing 1 ml. of bone solution were heated in a pressure cooker (Pentecon) at a pressure of 40 lb. per sq. in. (2·8 kg. per sq. cm.) for 6 hr. They were then opened and the contents treated in exactly the same way as the previous hydrolysate.

Determination of hexosamines.—This was done by a modification of the Elson-Morgan technique (Elson and Morgan, 1933). Aliquots of 1 ml. of the hydrolysate were taken and the hexosamines acetylated for 25 min. with an alkaline solution of acetyl acetone in stoppered tubes. The level of the water in which they were heated was the same as that of the solution inside the tube. After cooling, 1 ml. of ethyl alcohol was added to each, followed by 1 ml. Ehrlich's reagent. The intensity of the colour which developed was measured with an EEL portable colorimeter at 540 mμ. The experimental error was ± 10 per cent.

Determination of hydroxyproline.—This was carried out on 1 ml. aliquots of the hydrolysate by the method of Neuman and Logan (1950). The colour intensity was measured at 495 mμ. The experimental error was ± 5 per cent.

RESULTS

Macroscopic appearance of bones

In all cases the weight of the bones from the injected animals was less than those from the controls (Table I). This was most noticeable in the weanling rabbits when the percentage difference in weight was − 32·4. In the younger rabbits the percentage difference was − 22·5. Length was slightly affected in both these groups but not at all in the old group. No gross changes were noted in either the youngest or oldest group, but considerable changes were present in the weanlings. When the ends were sawn off and the marrow removed, bony protuberances

TABLE I.—*Weight and Length of Femurs at Death of Rabbits Injected with ^{90}Sr, and of Controls*

Mean age in days at injection.	Number of rabbits.	Dose μc./kg.	Mean survival days.	Wt. of bone (g.). Mean.	Max.	Min.	Length of bone. Mean.	Max.	Min.	Mean ratio/ Wt./L.
2	2	500	196	4·36	4·5	4·2	8·1	8·2	8·0	0·54
43	4	500–630	212	3·83	5·07	3·15	8·2	8·5	7·8	0·47
378	4	1000	19	6·24	6·95	5·58	8·4	8·1	8·8	0·74
2	1	—	196	5·63	—	—	9·1	—	—	0·62
43	4	—	212	5·67	5·88	5·46	8·9	9·0	8·8	0·64
378	4	—	197	6·9	7·2	6·62	8·5	8·8	8·4	0·81

into the marrow cavity were seen in the radioactive bones. In some cases these excrescences stretched right across the cavity. Both above and below these abnormalities, the bones exhibited a fragile " moth-eaten " appearance.

The difference in the rate of bone growth after injection produced such large differences in the weight in one age group between control and radioactive bones that it was difficult to find a satisfactory method of expressing the results of chemical analysis for purposes of comparison. The ratio of the concentration of the substance in mg./g. dry defatted bone powder to the concentration of calcium in mg./g., however, gave results which were little different from the actual concentrations in mg./g. dry defatted bone powder, so only the latter figure is given in the Tables. Calcium was chosen as the reference substance because it is characteristically present in large amounts.

Calcium analyses

There is no significant difference between the calcium content of the shafts of the irradiated and control bones in any age group (Table II), but in the ends there is more calcium present in the irradiated bones of the weanling group (Table III) and rather less in the end of the irradiated bones of the oldest rabbits. The calcium content of both irradiated and control bones in the older age groups is noticeably higher than in the youngest rabbits.

Nitrogen analyses

There appears to be a general rise in the amount of nitrogen present in both the shafts and the ends of the bones of the animals injected at 5–7 weeks of age (Tables II and III). In the younger age group there is no change in the shafts and a fall in the ends, while in the oldest animals the changes are not significant. The mean nitrogen/calcium ratio in the shaft of the irradiated bones in the weanling animals is 0·136 compared with 0·118 in the controls, showing an increase of 15·2 per cent in the irradiated bone.

Hexosamine analyses

The hexosamine is increased by a percentage change of + 52 in the shaft and + 33 per cent in the ends of the irradiated bones of rabbits aged 6–8 weeks when injected. The ratio of hexosamine to calcium is increased by + 55 in the shaft. There is no significant change in the hexosamine of the youngest and oldest rabbits irradiated (Tables II and III).

TABLE II.—*Chemical Analysis of Shafts of Femurs of Rabbits Injected with ^{90}Sr and Controls (mg./g. Dry Defatted Bone Powder)*

Mean age in days at injection.	Number of rabbits.	Dose. μc./kg.	Calcium. Mean.	Calcium. Max.	Calcium. Min.	Nitrogen. Mean.	Nitrogen. Max.	Nitrogen. Min.	Hydroxyproline. Mean.	Hydroxyproline. Max.	Hydroxyproline. Min.	Hexosamine. Mean.	Hexosamine. Max.	Hexosamine. Min.
2	2	500	259	260	258	33·2	34·0	32·5	21·7	21·9	21·6	2·04	2·05	2·03
43	4	500–630	271	277	258	36·9	42·8	31·6	20·2	21·8	19·2	2·94	2·99	2·87
378	4	1000	307	317	302	34·2	37·9	29·9	25·0	28·0	22·0	1·4	1·9	1·0
2	1	—	260	—	—	33·4	—	—	22·9	—	—	1·85	—	—
43	4	—	274	277	266	32·2	37·9	27·4	22·3	23·7	21·2	1·93	2·10	1·73
378	4	—	310·5	319	295	33·2	33·2	22·6	24·0	27·0	21·0	1·6	1·7	1·5

TABLE III.—*Chemical Analysis of Ends of Femurs of Rabbits Injected with ^{90}Sr and of Controls (mg./g. Dry Defatted Bone Powder)*

Mean age in days at injection.	Number of rabbits.	Dose μc./kg.	Calcium. Mean.	Calcium. Max.	Calcium. Min.	Nitrogen. Mean.	Nitrogen. Max.	Nitrogen. Min.	Hydroxyproline. Mean.	Hydroxyproline. Max.	Hydroxyproline. Min.	Hexosamine. Mean.	Hexosamine. Max.	Hexosamine. Min.
2	2	500	220	220	220	39·9	40·6	39·2	35·3	35·7	34·9	2·82	2·83	2·82
43	4	500–630	238	248	231	57·8	62·5	51·2	29·2	32·1	29·0	3·85	4·11	3·62
378	4	1000	266	272	262	54·3	56·7	51·7	36·0	37·0	35·0	3·90	4·50	3·00
2	1	—	228	—	—	51·8	—	—	33·9	—	—	2·66	—	—
43	4	—	199	206	189	44·9	47·1	41·5	32·4	36·0	23·6	2·90	3·38	2·64
378	4	—	283	311	270	52·0	57·4	42·8	37·0	39·0	26·0	4·00	5·0	3·1

Hydroxyproline analyses

The hydroxyproline present in the irradiated shafts of the weaning rabbits (Table II) is about 10 per cent lower than that in normal bones. The ratio of hydroxyproline to calcium shows a percentage change of − 8. From the hydroxyproline figures it is possible to calculate that proportion of the total nitrogen which is collagen nitrogen. The figures used for this calculation were : 14 per cent of collagen is hydroxyproline, 18 per cent of collagen is nitrogen (Eastoe and Eastoe, 1954) (assuming that the collagen in the radioactive bones is chemically identical with that in normal rabbits). It was found that approximately 20 per cent less of the total nitrogen was collagen nitrogen in the irradiated shafts compared with the normal shafts. The corresponding figure for the ends of the bones was 27 per cent. In the rabbits injected at 48 hr. of age and one year (Tables II and III) changes in hydroxyproline are probably not significant.

DISCUSSION

Certain points must be stressed before the results are discussed. It is extremely difficult to free the bones from marrow and tendon remnants before powdering. Consequently the nitrogen figures may be too high especially for the ends of the bones, where removal of marrow from the spongy bone is particularly difficult. Again, Hamilton (1952) maintains that 15–20 per cent of the total protein is soluble in water (this protein is probably a soluble form of collagen which may be its precursor), while Kellgren (1952, 1953–4) states that most polysaccharides are soluble in water to some extent, although he does not make it clear whether they are soluble when in combination with proteins. Protein and polysaccharides may therefore have been lost when the bone was ground and collected under water, a procedure which is essential when dealing with highly radioactive dust. On the other hand, the figures for the total nitrogen and hexosamines in normal bone agree well with those recorded by other workers (Rogers, 1949 ; Rogers, Weidmann and Parkinson, 1952 ; Eastoe and Eastoe, 1954).

Failure to clean the bones adequately is likely to be reflected particularly in the figures for total nitrogen : stress is not therefore laid on these figures nor on the calcium : nitrogen ratios which, however, in the weanling group suggest a rather lower degree of calcification of the irradiated bones. The calcium per gram of bone is not significantly different in irradiated and normal cortical bone. The absolute calcium figures for the ends of the bones of the rabbits injected when 6–8 weeks old is, however, definitely higher than the calcium figures for the controls. This is similar to the results found in rats when injected with radium (Evans, Harris and Bunker, 1944). The high degree of calcification in older bone found in the present investigation by chemical estimation has also been noted when measuring variations in calcification by densitometry methods (Owen, 1956).

The most striking change observed is the extremely high hexosamine content of the bones of animals injected when 6 weeks old both in comparison to the controls and to the other age groups. This age group also shows an effect on the hydroxyproline content. In view of what is known of the morphological effects of irradiation and the pattern of bone growth and therefore of strontium retention, the chemical changes are not surprising. Vaughan and Jowsey (1956) have reported that microradiological examination of certain long bones from the

same weanling rabbits as those used for some of these chemical analyses, apart from general retardation of growth have shown two consistent gross abnormalities, varying in degree. First an abnormal persistence of cartilage remnants due to failure of normal resorption and remodelling at one of the levels where ^{90}Sr retention is greatest ; second, the development of abnormal periosteal bone again at a level of high ^{90}Sr retention (Vaughan and Jowsey, 1956 ; Owen, Jowsey and Vaughan, 1955). Cartilage is rich in mucopolysaccharides and this persistence of cartilage in irradiated bone may well explain the high figure for hexosamine and the high hexosamine : calcium ratio noted.

On the other hand, the low figure for hydroxyproline in the irradiated bone may be a measure of the reduced collagen in the abnormal periosteal bone and the abnormal bone formed in ralation to the unresorbed cartilage (Owen and Vaughan, 1956). Alternatively, this figure together with the lower proportion of the total nitrogen which is collagen nitrogen, may indicate that collagen with a different amino acid pattern from the normal may be produced. If the theory that collagen is produced by the osteocytes is correct (Rutishauser and Majno, 1951 ; Jackson, 1953), then it seems possible that if these cells are injured by radiation from ^{90}Sr then their metabolism will be disturbed and a collagen with abnormal properties will be produced.

The present results suggest that there is a definite change in certain chemical constituents of the long bones brought about by ^{90}Sr injected into rabbits aged 5–7 weeks. No appreciable change is found in the long bones of rabbits injected when much younger or much older. This can probably be explained by the pattern of growth at the weanling age which controls both the uptake and retention of ^{90}Sr (Jowsey *et al.*, 1953 ; Jowsey, Owen, Tutt and Vaughan, 1955 ; Owen *et al.*, 1955). Weanling rabbits take up ^{90}Sr in high concentration in the region of the epiphyseal plate and in certain regions of endosteal and periosteal bone. Much of this strontium-containing tissue is not resorbed in the normal pattern of growth at this age and remains to cause radiation dysplasia (Vaughan and Jowsey, 1956). In the youngest rabbits strontium is taken up much more diffusely and also owing to the pattern of growth at this age much of the strontium-containing tissue is removed by normal remodelling. In the older animal growth is far less active and therefore less strontium is taken up initially and therefore less remains to cause radiation dysplasia.

SUMMARY

An analysis of calcium, total nitrogen, hydroxyproline and hexosamine in the bones of rabbits following a single injection of ^{90}Sr and in normal controls is reported.

In animals aged 6–8 weeks at the time of injection and killed approximately 6 months later, there are significantly larger amounts of hexosamine and lesser amounts of hydroxyproline both in absolute figures and expressed as a ratio to the calcium content in the irradiated bones than in the controls.

In animals aged 48 hr. or 1 year at the time of injection no great effect on weight, length and chemical composition is noted.

The effect at the weanling age is explained by the pattern of bone growth.

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THE EFFECT OF DIVALENT METALS ON THE STABILITY OF A TYPHOID BACTERIOPHAGE AND ITS REACTION WITH ITS RECEPTOR

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DIVALENT metals are implicated in the multiplication of many bacteriophages and appear to be concerned in most of the recognised stages of the growth cycle (Fildes, Kay and Joklik, 1953). Phage 1 of our collection, active on several strains of *Salmonella typhi*, requires calcium and fails to cause lysis unless it is added to the medium (Fildes, 1954). In the course of experiments on the reaction of this phage with its isolated receptor (Kay, 1955) it was noted that magnesium or calcium were needed to maintain the activity of the phage when suspended in phosphate buffer. Failure to add magnesium or calcium resulted in the complete loss of the phage within a few minutes at 37°. In the present paper the mechanism of this inactivation is examined and its relation to the reaction between the phage and its receptor is discussed.

MATERIALS AND METHODS

Bacteria

Salm. typhi R4 was used to assay the phage. *Salm. typhi* O 901 R was used to prepare the phage receptor. Both these organisms were described in a previous paper (Kay, 1955).

Bacteriophage

Phage 1 was grown and purified by differential centrifugation as described previously (Kay, 1955). It was usually suspended in phosphate buffer pH 7·6 (0·067 M) containing magnesium (0·002 M) and electro-dialysed gelatin (0·02 per cent) but when wanted in collidine buffer it was centrifuged again, washed in collidine buffer and re-suspended in the same diluent. Radioactive phage was made by growing the phage in medium containing ^{32}P (2 μc./ml.) as orthophosphate. Phage was assayed by the two-layer method using R4 as the background organism.

^{32}P was assayed in 5 ml. samples which were diluted to 10 ml. with water and counted in a 10-ml. liquid counter tube.

Collidine buffer

This consisted of collidine, re-distilled and kept under nitrogen, 0·05 M solution in water adjusted to pH 7·4 with acetic acid (Analar grade). I am indebted to Dr. G. G. F. Newton of this department for the details of this buffer. It has two properties which were of use in the present work. It is completely volatile and is therefore useful in electron microscopy and unlike phosphate buffer it does not form unionised complexes with calcium and magnesium.

Electron microscopy

Phage suspensions in collidine buffer were suitably diluted in the same buffer, placed on thin carbon films and allowed to dry in air. Aqueous suspensions of the phage could not be used as this procedure appeared to damage the particles by separation of the " head " and

" tail " parts. Nor could non-volatile buffers be used since it proved impossible to dialyse the salts away from the films without causing an apparent rupture of the phage " heads ". The dried specimens were shadowed with a gold-palladium alloy.

Phage receptor

This was isolated from *Salm. typhi* O 901 R by the hot water extraction procedure and purified as described previously (Kay, 1955). It was used as a suspension in water and diluted appropriately in water before use.

EXPERIMENTAL

Inactivation of Phage 1 *and its Prevention by Various Cations*

Tubes containing phosphate buffer pH 7·6 (0·033 M) and Ca-free gelatin (0·01 per cent) supplemented with a range of different cations at 0·0001 M (as chlorides or sulphates) were inoculated with phage 1 at 8×10^3/ml. and kept at 37°. After 40 min. each tube was assayed for phage remaining active. It was found that all phage had disappeared from the tubes containing buffer alone, beryllium, manganese, zinc, copper and ferric iron. All the phage had survived in the calcium tube, 70 per cent in strontium, 36 per cent in barium and 16 per cent in magnesium. Later experiments with magnesium showed that concentrations of 0·002 M or more completely protected the phage for 1 hr. at 37°. Attempts to recover the lost phage by addition of calcium were unsuccessful. It was at first thought that the phage had been lost by adsorption to the walls of the vessel but this was shown to be unlikely when incubation of the phage in paraffin wax-coated tubes and cellulose nitrate tubes also caused inactivation unless calcium was present. The possibility that the phage particles were suffering physical damage was next examined. The release of nucleic acid from inactivated phage was looked for and morphological changes in the phage particles were examined in the electron microscope.

Release of Nucleic Acid

Phage 1 (3×10^{11}/ml.) labelled with ^{32}P was diluted 1/20 with phosphate buffer (0·067 M) to reduce the magnesium concentration to 0·0001 M. It was then added to each of two tubes containing buffer (final concentration 0·033 M) one of which also contained calcium (0·0001 M). The residual magnesium, 0·00001 M was too small to prevent inactivation of the phage. The initial phage titre, the total ^{32}P and the unsedimentable ^{32}P after centrifuging at 20,000 r.p.m. were all determined on samples from the calcium tube. After keeping the tubes at 37° for 1 hr. the phage titres and unsedimentable ^{32}P were determined in both. The phage titres obtained and radioactivity measurements after correction for background are given in Table I.

TABLE I.—*Inactivation of Phage* 1 *and Release of* ^{32}P *after* 1 *hr. at* 37° *in Phosphate Buffer*

	Phage titre/ml. or ^{32}P counts/min./5 ml. Sample tested.	
	Calcium 0·0001 M.	No calcium.
Initial phage	$1·5 \times 10^9$	—
Final phage	$1·3 \times 10^9$	9×10^4
Total ^{32}P	244	—
Initial unsedimentable ^{32}P	71	—
Final unsedimentable ^{32}P	58	163

The figures show that inactivation of the phage (99·99 per cent) is accompanied by an increase in the amount of ^{32}P which is unsedimentable at 20,000 r.p.m. and therefore no longer part of the phage particles. After allowing for the initially unsedimentable ^{32}P (71 counts/min.) this amounts to 53 per cent of the ^{32}P which was initially sedimentable and which was part of the phage particles. The phage in calcium is not inactivated and does not show any release of ^{32}P. In another experiment 59 per cent of the ^{32}P became unsedimentable after inactivation of the phage. The U.V. absorption spectra of the sedimentable and unsedimentable fractions of the phage were examined and it was found that the latter had a marked absorption maximum at 260 mμ whereas the former showed none. The properties of the unsedimentable fraction were not examined further but from its phosphorus content and its absorption at 260 mμ it was assumed to be phage nucleic acid or its degradation products. It is considered therefore that phage 1 is inactivated in phosphate buffer by the loss of part at least of its nucleic acid.

Electron Microscopy of Phage 1 *and its Inactivation Products*

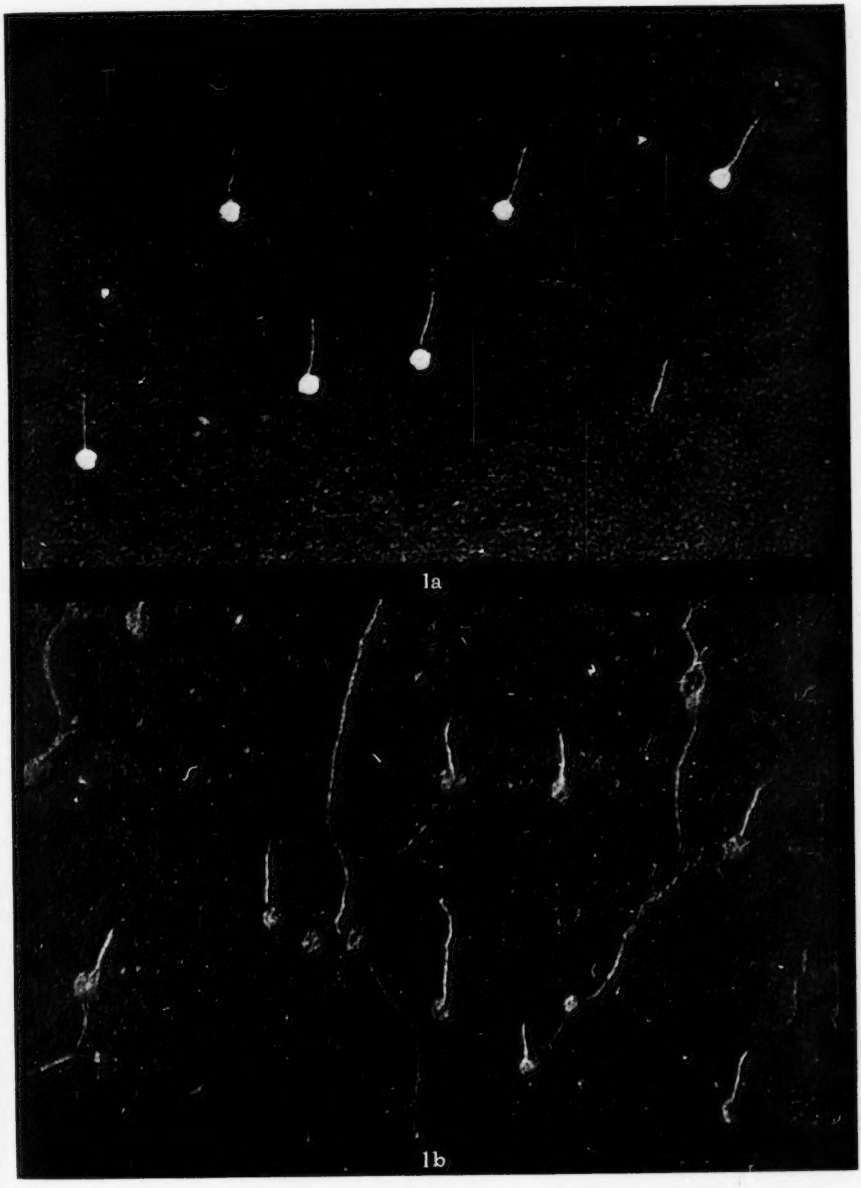
Phage 1 was examined in the electron microscope to determine its morphology in the active state and to observe any change that might occur during inactivation. The best pictures were obtained with the phage suspended in collidine buffer which evaporates from the films used in the microscope without leaving any solid matter. Fig. 1a shows the appearance of phage 1 particles. The " heads " are spherical, 80 mμ diameter, and the tails are 220 mμ long.

Attempts to inactivate the phage in collidine buffer at 37° were unsuccessful as will be described later, and it proved necessary to treat the phage with one of the calcium complexing agents, citrate, phosphate or " versene " (ethylenediamine tetra-acetic acid). Versene was chosen as the most suitable agent to inactivate the phage since it was the most active and would leave the least residue on the films. The addition of versene (sodium salt pH 7·6) at 0·0004 M to phage 1 in collidine buffer results in extremely rapid inactivation even at room temperature. The loss in activity is accompanied by the disappearance of the bluish turbidity of the phage suspension. The inactivated particles have an entirely different appearance from the active ones as is shown in Fig. 1b. The " heads " are now flattened or deflated as evidenced by the lack of shadow. Long filaments are seen in every field examined. Phage particles inactivated in phosphate buffer show a similar appearance to those inactivated by versene but the photographs were of poorer quality due to the phosphate on the film. Attempts to wash the inactivated phage free of phosphate reduced the particles to unrecognisable fragments. It is concluded that inactivation of the phage is accompanied by damage to its structure involving the escape of its nucleic acid and the consequent deflation of the head. Although the fine filaments seen in Fig. 1b could be nucleic acid which had been expressed from the tails of the phage particles the micrographs are not clear enough to be certain whether the nucleic acid did come out through the tail or through the damaged head membranes.

EXPLANATION OF PLATE.

FIG. 1a.—Phage 1 prepared in collidine buffer. Air-dried on carbon film and shadowed with gold-palladium. × 62,000.

FIG. 1b.—Phage 1 in collidine buffer inactivated by versene (0·0004 M). Air-dried on carbon film and shadowed with gold-palladium. × 62,000.



1a

1b

Inactivation of the Phage by Complexing Agents

The unexpected stability of phage 1 in collidine buffer compared with its instability in phosphate buffer suggested that the agent responsible for the breakdown of the phage was the phosphate. This is shown to be so by the results given in Table II which also includes the effect of citrate and versene on phage 1 in collidine buffer.

The phage ($1{\cdot}8 \times 10^4$/ml.) was kept at 37° in collidine buffer together with the additions mentioned in the Table in glass-stoppered tubes to prevent the evaporation of the collidine acetate which would occur through the normal cotton wool plug. Samples were taken at intervals and assayed for phage after dilution 1/10 in 2 per cent peptone water.

TABLE II.—*Inactivation of Phage* 1 *in Collidine Buffer by Complexing Agents*

Additions to buffer.	Percentage phage active. 2 min.	10 min.	20 min.
Buffer alone	100	100	100
Versene 0·0004 M	0	0	0
Citrate 0·0004 M	77	62	51
Phosphate pH 7·4, 0·025 M	0	0	0
,, 0·025 M + Mg 0·002 M	100	100	100

Phage 1 is completely inactivated by phosphate at 0·025 M and by versene at 0·0004 M. Citrate at 0·0004 M causes a slower inactivation. Magnesium at 0·002 M is seen to prevent completely the inactivation caused by the phosphate.

Inactivation of Phage 1 *by Receptor*

Previous experiments to determine whether phage 1 could react with its receptor isolated from the host bacteria in the absence of calcium were always vitiated by the instability of the phage in the phosphate buffer used. However, with the discovery that phage 1 is stable in collidine buffer this experiment became feasible. Five stoppered tubes were set up containing collidine buffer (plus 0·01 per cent calcium-free gelatin) alone, buffer and calcium (0·005 M), buffer and receptor, and buffer, receptor and two concentrations of calcium (0·005 M and 0·0005 M). Each was inoculated with phage in collidine buffer ($1{\cdot}1 \times 10^4$/ml.) and kept at 37°. Phage was assayed at intervals after dilution 1/10 in 2 per cent peptone water. The results are shown in Fig. 2. This shows that phage 1 is stable in collidine buffer and that it is inactivated by receptor when calcium is absent. An unexpected result is that calcium (0·0005 M) reduces the rate of inactivation and at 0·005 M almost inhibits it. It appears therefore that phage 1 can react with its receptor in the absence of calcium and is thereby inactivated. If, as suggested previously (Kay, 1955), inactivation by isolated receptor corresponds to the infective stage in the phage multiplication cycle in whole bacteria then phage 1 could probably adsorb to and infect its host in the absence of calcium. The actual experiment with growing bacteria has not been done yet because the phosphate normally present in the media used is sufficient to inactivate the phage. The calcium requirement of this phage is probably confined to the function of protecting the phage against the phosphate in the medium.

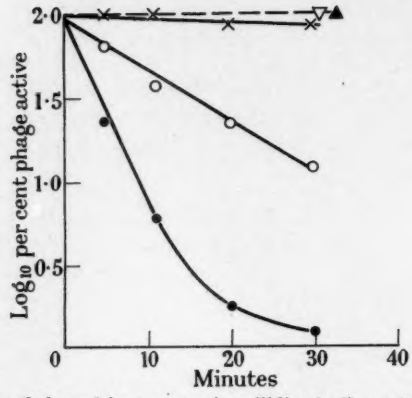


FIG 2.—Inactivation of phage 1 by receptor in collidine buffer at 37°. Effect of calcium.

●——● Receptor alone.
⊙——⊙ Receptor 0·0005 M-Ca.
×——× Receptor 0·005 M-Ca.
- - - -▲ Phage alone 0·005 M-Ca.
- - - -▽ Phage alone. No Ca.

DISCUSSION

It has been shown previously that phage 1 is caused to release its nucleic acid when it reacts with its isolated receptor (Kay, 1955) and it is now shown to release its nucleic acid also when treated with the calcium and magnesium complex-forming agents: phosphate, citrate and versene. It has been shown that the inactivation of the phage by either phosphate or receptor is prevented by magnesium or calcium. This effect could be accounted for by assuming the phage to be a calcium (or magnesium) complex and the bacterial receptor to have the property like the phosphate, citrate and versene of forming poorly ionised complexes with the calcium. The action of the complexing agent in each case is to remove the calcium from the phage and leave it in an unstable state which readily releases its nucleic acid. The addition of calcium ions to the medium would affect the equilibrium between the stable and unstable forms of the phage in favour of the stable form and so reduce its likelihood of being deprived of calcium by the receptor or the phosphate and consequent inactivation. Adams (1949) working with coli phages noted that the inactivation which took place in the presence of certain concentrations of sodium ion could be prevented by magnesium and proposed a stable magnesium-phage complex in equilibrium with an unstable magnesium-less phage to explain this. However, he did not take into account the effect of the small amount of phosphate present in all his experimental media. This may have been the major cause of phage inactivation as in the present case of the typhoid phage.

The rôle of calcium in the multiplication of phage 1 is now seen to be an indirect one since it is probably confined to preserving the phage against inactivation by one of the normal constituents of the medium, phosphate. In order to prove that calcium is not needed in the multiplication of the phage it would be necessary to grow the host bacteria in a medium containing insufficient phosphate to inactivate the phage. This introduces difficulties in maintaining the pH of

the medium constant since phosphate buffer at 0·05 M is necessary in a culture of R4 growing in a synthetic medium containing glucose. Attempts are being made to overcome this by constantly adjusting the pH in a medium containing minimal phosphate and by using buffer systems other than phosphate.

SUMMARY

A typhoid phage is inactivated by phosphate, citrate and versene (ethylenediamine tetra-acetic acid) which have the common property of complexing divalent metals. Calcium, magnesium and to a lesser extent strontium and barium protect the phage against inactivation. Inactivation is accompanied by the loss of nucleic acid from the phage and the " deflation " of its " head " as seen in the electron microscope. The isolated phage receptor inactivates the phage in the absence of calcium when examined in collidine buffer in which the phage is stable, but calcium tends to prevent the action of the receptor. It is suggested that the phage is itself a complex of calcium (or magnesium) ions and that the complex formation of these cations with the bacterial receptor causes the phage to become unstable and is the mechanism by which the phage is caused to release its nucleic acid into the host cell.

The author wishes to thank Sir Paul Fildes, F.R.S., Director of the Unit, for his interest and encouragement during the course of the work, and Miss J. Sampson for the electron micrographs.

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FURTHER ATTEMPTS TO TRANSFER TRANSPLANTATION IMMUNITY BY MEANS OF SERUM

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IT has been established beyond question that serum antibodies, demonstrable by haemagglutination (Gorer, 1947) or by nephelometric methods (Bollag, 1956), are formed in response to the grafting of homologous tissues ; but there is strong evidence that, at least as far as serum haemagglutinins are concerned, these are not the operative agents of transplantation immunity (Mitchison and Dube, 1955 ; Kaliss, 1957). Attempts to transfer a state of heightened resistance against homografts of skin, using serum from specifically immunised mice, have so far been uniformly unsuccessful. Even regimens of daily or two-daily intraperitoneal inoculations of relatively large dosages of " immune " serum have failed to shorten the survival time of skin homografts in mice (Billingham, Brent and Medawar, 1954). The recent experiments of Voisin and Maurer (1956), while confirming that immunity may be transferred with cells, have given equivocal results with homologous " immune " serum.

The purpose of this paper is to report on an extension of our earlier work in mice. Not only has an attempt been made to evoke a more vigorous and generalised immunological response from the serum donors, but special attention has been paid to the possibilities : (1) that the dosages of serum used in previous experiments might have been too high ; (2) that it might be advantageous to begin treatment of the secondary hosts several days before transplanting to them their test-grafts ; and (3) that the serum might be more effective if administered intravenously rather than intraperitoneally.

MATERIALS AND METHODS

The design of the present experiments was exactly as before (Billingham, Brent and Medawar, 1954). After immunisation of mice (primary hosts) of one inbred strain with tissues from animals of a donor strain, the serum was prepared from the pooled blood of the primary hosts—obtained by cardiac puncture—and then injected into normal adult mice (secondary hosts). Primary and secondary hosts were always members of the same inbred strain. The capacity of the serum to confer heightened resistance (*i.e.*, immunity) on the secondary hosts was assessed in terms of the reaction of the recipients against skin homografts from the donor strain. Curtailment of the grafts' survival times compared with those of grafts on normal untreated animals, combined with certain other diagnostic features, would have shown that immunity had been transferred by the serum.

The mice used in these experiments belonged to the sublines of the Strong A and CBA strains, for which accurate transplantation data have already been obtained (see Billingham, Brent, Medawar and Sparrow, 1954). The primary hosts were invariably immunised three times, at three-weekly intervals, in order to exact a maximal response. Each immunisation entailed the intraperitoneal and subcutaneous injection of living spleen and kidney cells

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from the donor strain, the subcutaneous injections being carried out in widely separated areas in the hope that this would activate most of the lymph nodes. The fact that the regional lymph nodes are known to be primarily responsible for the destruction of homografts (Mitchison, 1954, 1955*a*; Billingham, Brent and Medawar, 1954) appears to justify such an approach. In addition, the first immunisation included the transplantation of a skin graft from the donor strain. In other words, the greatest possible care was taken to bring about the hyperimmunisation of the serum donors, which were bled on the 8th day after the final immunisation. In order to obviate any possible deleterious effects of storage, fresh serum was used almost invariably. This entailed careful planning of the immunisations so that groups of primary hosts became available for bleeding on the appropriate days. On the very few occasions on which stored serum was used it had been preserved at 4° for periods never exceeding 48 hr.

RESULTS

The results are summarised in the Table. None of the experiments indicates

TABLE.—*Attempts to Transfer Immunity directed against Skin Homografts by means of Serum*

Strain combination.	Experiment.	Dose of serum per day of administration (ml.).	Total dose (ml.).	Days of administration.*	No. of secondary hosts.	Appraisal of test grafts.
A → CBA	1	0·25	0·25	0	7	No immunity
	2	0·25	0·5	4, 6	4	,, ,,
	3	0·25	0·75	−1, 0 6	10	,, ,,
	4	0·25	1·25	−2 0, 2, 4, 6	3	,, ,,
	5	0·5	1·5	−2 0, 2	3	,, ,,
	6	0·25	1·5	−4, −2 0, 2, 4, 6	2	,, ,,
CBA → A	7†	0·25	0·25	0	5	,, ,,
	8	0·25	1·0	−2 0, 2‡, 4‡	4	,, ,,

* Day of transplantation = 0.
† Grafts treated with immune serum *in vitro* for 6 hours before transplantation
‡ Injections on these days were made intravenously.

that " immune " serum transferred a state of immunity to secondary hosts. These results, considered together with our previous findings, show that the outcome of the experiments was not affected by (*a*) variation over the range 0·25 ml. to 1·5 ml. in the total amount of serum administered, (*b*) the injection of the immune serum over a period beginning as early as 4 days before the transplantation of the test-grafts and ending as late as the day of graft biopsy, and (*c*) the use of the intravenous as well as the intraperitoneal route of injection. Equally ineffective was the prolonged impregnation of very thin grafts with " immune " serum before transplantation, combined with the intraperitoneal inoculation of the host with serum on the day of grafting (experiment 7).

It should, however, be mentioned that the grafts on some of the serum-treated animals displayed outward signs of vascular dilatation and congestion when examined on the 6th post-operative day. This effect was transient and clearly of minor importance to the well-being of the grafts, the survival times of which were not in the least curtailed.

One experiment, not included in the Table, made use of two CBA mice which had previously been made tolerant of A strain tissues (see Billingham, Brent and Medawar, 1956) and which bore A strain skin grafts of 100 days' standing.

Although such animals are themselves unable to react against their grafts, they are nevertheless capable of mediating a state of immunity which may be conferred upon them by the injection of immunologically activated cells. Tolerant mice are therefore ideal subjects for experiments on the passive transfer of immunity with serum. Each mouse received 2·0 ml. of " immune " serum, in divided doses, over a period of 8 days. In one of them the serum was injected intraperitoneally, in the other both intraperitoneally and intravenously. At no stage did the grafts on either mouse reveal the slightest sign of an immunological reaction such as might be expected had the serum contained cytotoxic antibodies.

DISCUSSION

Evidence of the protective action of homologous immune sera has been derived almost exclusively from work on mouse leukaemia. That serum from hyper-immunised donors will protect mice against certain leukaemic cells, provided that cells and serum are first incubated *in vitro*, has been known for some considerable time (Gorer, 1942 ; Mitchison, 1955*b*) ; it has recently been extended to suspensions of Sarcoma 1 cells (Mitchison and Dube, 1955). Gorer and Amos (1956) and Amos (1957) have also shown that it is possible to bring about a true passive transfer of immunity against certain leukaemic tumours, *i.e.*, by the simultaneous injection of the immune serum and the leukaemic cells into the hosts *without* prior incubation. Similar results have now been obtained with the Walker 256 rat carcinoma (Sekla and Barvič, 1956).

As far as the transplantation of *normal* homologous tissues is concerned, the only evidence lending support to the view that protective antibodies are present in " immune " sera—apart from the rather equivocal results of Voisin and Maurer (1956)—lies in the impairment of the proliferative capacity of rabbit skin epithelial cells after their prolonged incubation *in vitro* with the serum from specifically immunised rabbits (Billingham and Sparrow, 1954). However, this effect is too feeble to account convincingly for the violence of the homograft reaction *in vivo*.

The present experiments were designed to test the ability of serum from immunised mice to transfer an immunity measurable by the precocious breakdown of homologous skin grafts, and possible shortcomings of previous attempts on these lines (Billingham, Brent and Medawar, 1954) were carefully avoided. As before, the results have been uniformly negative. It is therefore impossible to escape the conclusion that the sera of these mice did not contain protective antibodies directed against the homologous skin grafts. Indeed, the work of Algire, Weaver and Prehn (1954) and Weaver, Algire and Prehn (1955) has shown that a variety of homologous tissues (including some tumours) remain unharmed when exposed in diffusion chambers to the body fluids of specifically immunised mice, provided that the ingress of host cells is completely prevented. It is also well established that transplantation immunity in respect of both tumour tissue (Mitchison, 1954, 1955*a*) and skin (Billingham, Brent and Medawar, 1954) can be " adoptively " transferred with living cells from the draining lymph nodes and spleens of immunised mice. Our own negative results with serum further support the view that the homograft reaction against normal tissues, like tuberculin sensitivity and other allergic responses of the delayed type, is mediated by cells but not by serum (also see review by Brent, 1957).

It is at present not possible to put forward a satisfactory explanation for the contradictory results obtained with homografts of skin and a variety of solid

tumours on the one hand, and with dissociated leukaemic cells on the other. It is not inconceivable that they may be due to unique properties of the leukaemic tumours (Gorer and Amos, 1956), and the dissociated state of the tumour may well be of crucial importance. Yet Sekla and Barvič's (1956) findings with the Walker rat carcinoma appear to make such an explanation rather less acceptable.

SUMMARY

Further attempts have been made to transfer immunity passively against skin homografts with serum from hyperimmunised mice. Variations in serum dosage, regimen of administration relative to the time of skin grafting, and route of injection have all given uniformly negative results. This finding is in agreement with the thesis that the agent of skin homograft destruction is not present in the serum of immune animals, and that transplantation immunity is transferable with cells only.

The helpful criticism of Professor P. B. Medawar, F.R.S. is gratefully acknowledged.

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THE PIGMENTATION OF *PASTEURELLA PESTIS* ON A DEFINED MEDIUM CONTAINING HAEMIN

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ISOLATED cells of *Pasteurella pestis* will not grow under aerobic conditions unless haemin or some other substance capable of destroying hydrogen peroxide is added to the medium (Herbert, 1949).

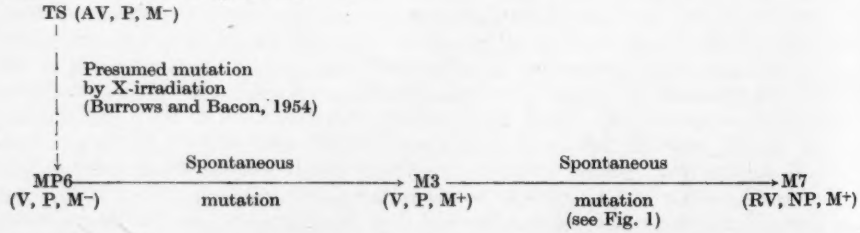
Using a concentration of haemin greatly in excess of the minimum requirement for growth, we have observed an additional effect. On a suitable medium, under well-defined conditions, many strains of *P. pestis* produce dark brown colonies, while others produce colonies lacking the brown pigment. After prolonged incubation, non-pigmented secondary colonies usually develop from pigmented colonies (Fig. 1). Our interest in this phenomenon was greatly increased when we observed that a pure non-pigmented strain, isolated from the secondary growth of a highly virulent pigmented strain, displayed reduced virulence for mice. It seemed that this phenomenon merited further investigation.

This paper is concerned with the development of a medium giving maximum differentiation between pigmented and non-pigmented strains, the chemical nature of the pigment and a preliminary survey of the pigmentation characteristics of a number of strains. The reduced virulence of non-pigmented mutants of virulent strains will be dealt with in a subsequent paper.

MATERIALS AND METHODS

Strains

The strains mostly studied were labelled TS, M3 and M7. TS is a convenient abbreviation for "Tjiwidej" smooth, a classical protective avirulent (AV) strain (Otten, 1936). It is pigmented (P) and requires methionine for growth (M^-). M3 is highly virulent (V), pigmented, and independent of methionine (M^+). M7 is also methionine-independent, but is non-pigmented (NP) and has reduced virulence (RV). The relationship between these strains is shown in the following scheme:



Non-pigmented mutants were also readily selected directly from MP6. Dependence or independence on methionine did not appear to affect the characters controlling pigmentation or virulence.

Other strains examined comprised a collection made in this Establishment from numerous sources.

Cultures and suspensions

Strains were stored as dried cultures, prepared by the method of Stamp (1947). For routine use, cultures were prepared by incubating in casein acid hydrolysate-galactose liquid medium (Ross, Hakes and Herbert, unpublished) for 48 hr. at 28°, with shaking. These cultures were stored at 2° for up to one month; full viability was maintained throughout this period.

Inocula for pigmentation tests were taken from these liquid cultures. Dilutions were made in phosphate buffer, pH 7·4.

Basal chemically-defined medium

The basal medium, on which the difference in colonial pigmentation between strains was first observed, had the following composition (for approximately 1 litre).

KH_2PO_4, 4·12 g. + 300 ml. water, adjusted to pH 6·8 or 8·0 with N-NaOH; glucose, 2·24 g. (3·7 ml. of a 60 per cent solution); NH_4Cl, 0·53 g. and $(NH_4)_2SO_4$, 0·53 g. dissolved in 100 ml. water: DL-phenylalanine, 0·12 g. (30 ml. of a 0·4 per cent solution); DL-methionine, 0·06 g. (5 ml. of a 1·2 per cent solution); L-cystine, 0·048 g. (10 ml. of a 0·48 per cent solution); DL-glutamic acid, 0·132 g. (10 ml. of a 1·32 per cent solution); glycine, 0·05 g. (5 ml. of a 1·0 per cent solution); DL-valine, 0·048 g. (5 ml. of a 0·96 per cent solution); L-leucine, 0·027 g. (12·2 ml. of a 0·224 per cent solution); DL-isoleucine, 0·055 g. (15 ml. of a 0·363 per cent solution); DL-serine, 0·020 g. (5 ml. of a 0·4 per cent solution); DL-threonine, 0·048 g. (5 ml. of a 0·96 per cent solution); pantothenate, 0·25 ml. of a 0·1 per cent solution of the calcium salt; biotin, 1 ml. of a solution containing 1·25 mg./l.; haemin, 0·025 g. (10 ml. of a 0·25 per cent solution in 0·01 N-NaOH); $MgSO_4.7H_2O$, 0·1 g. (10 ml. of a 1 per cent solution); $ZnSO_4.7H_2O$, 1 ml. of a 0·01 per cent solution; $MnSO_4.7H_2O$, 1 ml. of a 0·01 per cent solution; lactic acid, 1 ml. of a 4 per cent v/v solution; agar, 20 g. + 500 ml. water, adjusted to pH 7 with N-NaOH.

Solutions were sterilised by autoclaving except those of glucose and L-cystine which were sterilised by filtration. The solutions of ammonium salts, buffer and agar were pooled immediately after autoclaving and the mixture was stored in screw-capped glass bottles at room temperature. When plates were required, bottles of the mixture were melted in a steam bath and the other constituents were added immediately before pouring. The pH values quoted for media are those to which the buffer was adjusted before mixing with the other constituents.

Spectroscopic measurements

These were made with the Hartridge reversion spectroscope.

RESULTS

Development of a medium giving high pigmentation

Modifications of the basal medium were made to increase the differentiation of pigmented and non-pigmented strains. The variables considered were nutrients, pH of medium, temperature of incubation and haemin concentration. The pigmented strain TS and the non-pigmented strain M7 were used throughout these tests.

It was found that the intensity of pigmentation of TS increased as the haemin concentration was raised from the original level of 25 μg./ml. to 100 μg./ml. The higher level was used thereafter.

Growth of isolated cells was entirely reliable at 28° on media of pH either 6·8 or 8·0, but was uncertain at 37°, especially at the higher pH. At 28°, pigmentation of TS was inferior at the lower pH, while at 37° M7 showed some tendency to pigment. It was therefore decided to use media of pH 8·0 at 28°. High glucose

concentration greatly improved the reliability of growth at 37° (3 per cent glucose seemed to be optimum, compared with the standard concentration of 0·2 per cent). This advantage was offset, for our purposes, by the tendency of M7 to become somewhat pigmented at high glucose concentrations.

Further increase in the pigmentation of TS was achieved by eliminating some of the amino acids from the medium. Those retained were phenylalanine, methionine and cysteine, which were generally essential for growth of TS, threonine, which greatly stimulated growth and was essential for satisfactory pigmentation, and valine and isoleucine, which further augmented growth and pigmentation (they were essential for growth at 37°). Ammonium salts did not appear to affect pigmentation, but their omission led to very slow growth and small colony size.

In the presence of the six above amino acids, the effect of substituting other sugars for glucose was investigated, using sugar concentrations of 2 g./l. This had no effect on pigmentation: the difference between TS and M7 was equally obvious with glucose, galactose, xylose, fructose, maltose or mannose. The colonies were inclined to spread with glucose, fructose, maltose or mannose, but much less so with galactose or xylose. Although the connection is not clear, this difference in colonial morphology parallels observations on the sugar metabolism of *P. pestis* in liquid medium (Ross, Hakes and Herbert, unpublished). Much more acid is produced with glucose, fructose, maltose or mannose than with galactose or xylose. The more compact type of colony was preferable for our work, and galactose was therefore chosen for use as routine carbon source.

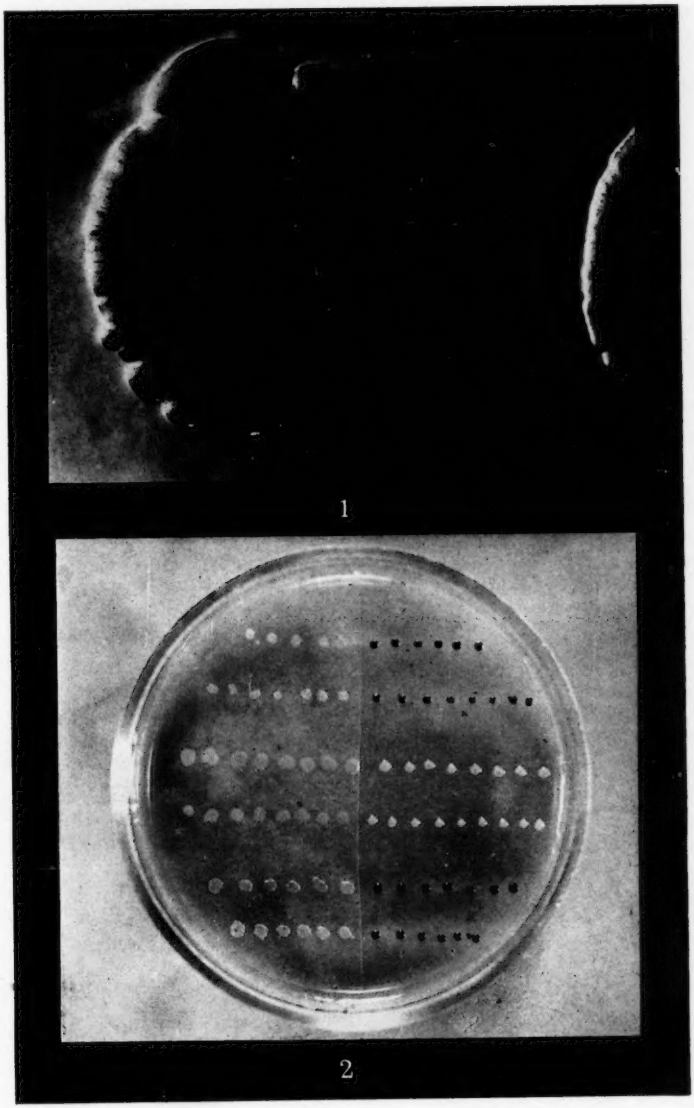
Subsequent to this change of carbon source, it was observed that glycine was a completely satisfactory alternative to threonine for stimulation of growth and promotion of pigmentation. The substitution of glycine for threonine was the final modification made in devising a medium suitable for reliable growth of TS and M7 and for clearly demonstrating the pigmentation of TS. On this medium, of which the composition is given in full below, colonies could be counted after 2 or 3 days' incubation at 28°, and the pigmentation of TS was clearly established in 3 or 4 days. M7 remained non-pigmented for about 8 days: on more prolonged incubation some pigment did appear, but the intensity of pigmentation never approached that of TS.

The composition of *ca.* 1 litre of the final pigmentation medium was; KH_2PO_4, 4·12 g. + 300 ml. water adjusted to pH 8·0 with N-NaOH: galactose, 2 g. (10 ml. of a 20 per cent solution): NH_4Cl, 0·53 g. and $(NH_4)_2SO_4$, 0·53 g. dissolved in 100 ml. water: DL-phenylalanine, 0·12 g. (30 ml. of a 0·4 per cent solution): DL-methionine, 0·06 g. (5 ml. of a 1·2 per cent solution): L-cysteine hydrochloride, 0·048 g. (5 ml. of a 0·96 per cent solution): DL-valine, 0·048 g. (5 ml. of a 0·96 per cent solution): DL-isoleucine, 0·055 g. (15 ml. of a 0·363 per cent solution): glycine, 0·05 g. (5 ml. of a 1·0 per cent solution): haemin, 0·10 g. (40 ml. of a 0·25

EXPLANATION OF PLATE

FIG. 1.—Pigmented colony with non-pigmented outgrowths. M3 colony after 12 days' incubation at 28° (M7 was cultured from the secondary growth). × 12·5.

FIG. 2.—Dependence of pigmentation on haemin. Petri dish containing pigmentation medium with left half overlayed with "Cellophane," inoculated with droplets of suspensions of 10^8 bacteria per ml. and incubated at 28° for 4 days. The impermeability of "Cellophane" to haemin, while not affecting growth, completely prevents pigmentation of M3 (top two rows) and TS (bottom two rows); M7 (middle two rows) is not pigmented even when haemin is accessible.



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Jackson and Burrows.

per cent solution in 0·01 N-NaOH) : $MgSO_4.7H_2O$, 0·1 g. (2·5 ml. of a 4 per cent solution) : $ZnSO_4.7H_2O$, 1 ml. of a 0·01 per cent solution : $MnSO_4.4H_2O$, 1 ml. of a 0·01 per cent solution : lactic acid, 1 ml. of a 4 per cent v/v solution : agar, 20 g. + 500 ml. water, adjusted to pH 7 with N-NaOH.

This medium was found suitable for the growth of the majority of the strains tested, but a few strains required arginine in addition. To permit growth of these strains, a solution of 104 mg. arginine hydrochloride in 10 ml. water was added. This had no marked effect on the behaviour of other strains.

Chemical nature of the pigment

From an early stage of the investigation, it was clear that pigmentation was dependent on the presence of haemin in the medium. This was readily demonstrated by the following experiment.

One half of a plate of pigmentation medium was covered with a semi-circular sheet of " Cellophane ", the other half being left uncovered. Droplets of TS, M7 and M3 suspensions were seeded across the plate, at right angles to the straight edge of the " Cellophane " sheet, and the plate was incubated at 28°. The " Cellophane ", which is impermeable to haemin, had no effect on growth, but entirely prevented the pigmentation of TS and M3. Pigmentation was normal on the uncovered half of the plate, showing that absorption of haemin from the medium was essential for pigmentation (see Fig. 2).

The texture of pigmented colonies was very much harder and drier than that of non-pigmented colonies. The latter readily gave a homogeneous suspension on shaking with buffer, but this was very difficult to achieve with pigmented colonies. These were easily washed off agar surfaces, but tenaciously retained their structure. Vigorous agitation resulted in free suspension of some of the bacteria, but the majority remained bound together in quite hard flakes. This effect was very striking with confluent growths (from heavy inocula), from which most of the bacteria could be washed off as a continuous sheet, brittle enough to be broken into small flakes by shaking. Under the microscope, wet preparations made from pigmented bacteria shaken with buffer were strikingly heterogeneous. Some free organisms appeared colourless, but the majority were bound together in closely packed masses, in which the cell walls were markedly thickened and yellow in appearance, the colour presumably being due to absorbed haemin.

Whether haemin was bound to bacterial substance after absorption was uncertain. However, it could be demonstrated experimentally that a large amount of haematin was recoverable from pigmented TS. A heavy suspension of TS was prepared after 6 days' growth on pigmentation medium, and the organisms dissolved by heating with N-NaOH. Following reduction with sodium hydrosulphite, an absorption band at 553 mμ wavelength was observed on spectroscopic examination. Addition of pyridine intensified the band, and led to the observation of a second band at 523 mμ. With M7 bacteria treated in the same way only a faint absorption at 553 mμ occurred. The bands observed with TS closely corresponded to those of pyridine haemochromogen, which, in our controls, prepared from haemin, appeared at 555 and 525 mμ. The pink colour of the complex was visible to the naked eye in the TS preparation and in the haemin control, but not in the M7 preparation.

These results were further confirmed by spectroscopic observations after the addition of potassium cyanide to dissolved bacteria. With TS, a faint cyanhaema-

tin absorption band was observed at 547 mμ, corresponding to that in the haemin control. In both cases, reduction with sodium hydrosulphite gave a visible pink colouration, and the absorption bands of reduced cyanhaematin, a very strong one at 535·5 mμ and a somewhat weaker one at 566 mμ, were observed. With M7, there was no detectable absorption before reduction, and only very faint absorption in the region of 534–537 mμ after addition of hydrosulphite.

It was clear that the pigmentation of TS was due to absorption and retention of haemin. The failure of M7 to show comparable pigmentation might have been due either to failure to absorb haemin or to destruction of haemin after absorption. A test of these alternative hypotheses was made by measuring the change in haemin concentration of liquid pigmentation medium when exposed to suspensions of M3 and M7 bacteria. It is clear from the results (Table I) that M7 absorbed haemin poorly compared with M3, which would satisfactorily account for their difference in pigmentation on our medium.

TABLE I.—*Absorption of Haemin from Liquid Pigmentation Medium by Suspended Bacteria*

Bacteria in suspension.	Haemin concentration (μg./ml.) in supernatant liquid after rotation at 28° for—	
	4·5 hr.	23 hr.
None	98	98
	99	100
M3	25	17
	28	17
M7	73	75
	73	73

Organisms harvested from growth on pigmentation agar medium for 5 days at 28° were suspended to a density of *ca.* 10^{10} organisms per ml. in liquid pigmentation medium contained in 4¼ in. × ½ in. (10·8 × 1·3 cm.) glass tubes. The suspensions were rotated at 28° for the times shown and haemin in the supernatants, after centrifugation, was estimated colorimetrically, using the EEL portable colorimeter with No. 621 blue filter.

Survey of a collection of strains of Pasteurella pestis *for ability to produce pigmented colonies*

Tests of ability to produce pigmented colonies were made for a number of strains of varied origin which had been stored as dried cultures (Stamp, 1947) for 2 years or more, and subsequently freeze-dried in "*Mist. desiccans*" a mixture of one part of broth and three parts of serum, with 7·5 per cent glucose added (Fry and Greaves, 1951). The freeze-dried cultures were suspended in broth, and streaked on pigmentation agar supplemented with arginine (essential for four of the strains). After incubation for 4 days at 28°, it was obvious that most of the cultures were heterogeneous, giving rise to a mixture of pigmented (P) and non-pigmented (NP) colonies. A suitable region of each plate, containing 50 to 100 discrete colonies, was marked off, and the numbers of colonies of each type in this region were counted. The results are compiled in Table II. On sub-culture, P colonies again gave mixtures, usually with P colonies in the majority, and with NP colonies mostly appearing as secondaries. In contrast, sub-cultures from NP colonies were, invariably, homogeneously NP.

TABLE II.—*Pigmentation of Different Strains of* Pasteurella pestis

		Percentage of pigmented colonies.
Avirulent strains	A1122	98
	EV76	0
	53H1	41
	Java	0
	14	23
	Soemedang	100*
	Tjiwidej	100*
Virulent strains	F9581	20
	I-72	25
	L27	100
	L36	98
	L37	100*
	139L	81
	New Mexico 499559	100*
	„ „ 499668†	93
	Shasta	100
	WCDCPp5A2258†	36
	„ 6†	98
	„ 54†	90
	Yokohama	15

* Few NP colonies among dense growth.
† Arginine-dependent strains.

DISCUSSION

Of twenty strains tested (after prolonged storage) only four gave populations homogeneous in pigmentation character, two strains being homogeneously P and two homogeneously NP. The other sixteen strains gave mixtures of P and NP colonies.

Mutation from P to NP occurred in all the strains which we have examined, but the reverse mutation, from NP to P, was never observed. It is therefore probable that all strains showing heterogeneity were originally of P rather than of NP type. In many cases, NP type predominated in heterogeneous populations, suggesting that this type was preferentially selected during culture or storage *in vitro*.

The classification of strains of *P. pestis* into the two types, P and NP, on the basis of high or low haemin absorption, was quite unequivocal under our conditions. In addition to haemin, P strains absorbed basic dyes from liquid medium more efficiently than did NP, suggesting that the mechanism responsible for ability to absorb haemin was not specific for this substance. While P and NP strains harvested from pigmentation medium differed widely in ability to absorb haemin from liquid medium (Table I) this difference was considerably less evident with organisms harvested from other media (*e.g.*, tryptic meat agar). P and NP strains thus appear to differ quantitatively in their ability to absorb haemin, and growth on pigmentation medium serves to enhance this quantitative difference.

Preliminary tests have shown that NP mutants of virulent strains generally have reduced virulence for mice. Mutation to NP type followed by preferential selection may therefore contribute to loss of virulence in laboratory cultures.

While loss of ability to pigment leads to reduction in virulence, possession of the ability obviously does not, by itself, confer high virulence. Ability to pigment

appears to be one of a number of properties collectively determining high virulence in *P. pestis*. Further, it is evident that ability to pigment is not connected with ability to resist phagocytosis, which sharply differentiates virulent from the majority of protective avirulent strains (Burrows, 1955).

Investigations of the changed virulence of NP strains, which are reported in a subsequent paper, indicate that such strains are population-limited *in vivo* by iron deficiency. It is tempting to speculate that the low ability to absorb haemin characterising such strains *in vitro* is, in some way, instrumental in producing the limitation observed *in vivo*.

SUMMARY

Many strains of *P. pestis*, when cultivated on a chemically defined medium containing haemin, form dark brown colonies. The dark brown pigmentation results from absorption of haemin from the medium. After prolonged incubation, pigmented colonies produce non-pigmented secondary colonies, which on sub-culture give only non-pigmented colonies. Pigmented and non-pigmented strains can most easily be differentiated on a medium containing galactose, amino acids and 100 μg./ml. of haemin, buffered at pH 8·0 with phosphate and incubated at 28°.

Of 20 laboratory strains tested after prolonged storage, 16 consisted of mixtures of pigmented (P) and non-pigmented (NP) types. On sub-culture, P strains gave predominantly P type with some NP mutants, but NP strains remained pure NP. In nature *P. pestis* is probably P type, and NP organisms accumulate during culture or storage *in vitro*.

NP mutants derived from P virulent strains are of reduced virulence for mice.

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THE VIRULENCE-ENHANCING EFFECT OF IRON ON NON-PIGMENTED MUTANTS OF VIRULENT STRAINS OF *PASTEURELLA PESTIS*

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MANY strains of *Pasteurella pestis* form pigmented colonies by absorption of haemin when grown on a suitable synthetic medium, as described in the foregoing paper. Non-pigmented secondary colonies subsequently appear, from which non-pigmented mutant strains can be cultivated. Non-pigmented mutants of virulent strains have reduced virulence for mice (corresponding mutants derived from avirulent strains are avirulent).

This paper deals with the peculiar virulence patterns of non-pigmented strains and the specific effect of iron compounds on them.

MATERIALS AND METHODS

Strains and cultures

As described in the foregoing paper.

Virulence tests

White mice (Porton strain) weighing 18–22 g. were used throughout.

Bacteria for injection were grown on tryptic digest meat agar slopes for 17 hr. at 28°, and then rotated for 3 hr. in tryptic digest meat broth at 37° (Burrows, 1955). They were then sedimented by centrifugation and re-suspended in phosphate buffer, pH 7·4.

The effect of additive substances was tested by dispersing them in arachis oil, using mechanical agitation with beads to produce an even suspension of small particles, and injecting 0·1 ml. of this suspension with 0·1 ml. of bacterial suspension.

Animals were observed for 14 days.

RESULTS

The virulence of strain M7

Strain M7 was derived from a non-pigmented (NP) sector developing on a pigmented (P) colony of the fully virulent strain M3. In contrast to its parent, M7 was not consistently lethal to mice injected with doses over the range 10^1–10^7 organisms. Such doses resulted in the death of approximately half the injected animals, and there was no proportionality between numbers of deaths and numbers of injected organisms. Deaths from M7 infection usually occurred later than from infection with M3. The results of a number of virulence titrations of strains M3 and M7 are summarised in Table I which illustrates the different virulence patterns of the two strains.

There was no enhancement of virulence of M7 after mouse passage, and all organisms recovered from mice dead from M7 infection were non-pigmented; back mutation from NP to P was not observed either *in vivo* or *in vitro*.

TABLE I.—*Virulence of Strains M3 and M7 of* Pasteurella pestis

Dose (number of organisms).	Deaths per 10 mice. M3.	Deaths per 10 mice. M7.
10^1	6	0 to 1
10^2	10	4
10^3	10	4 to 5
10^4	10	3 to 4
10^5	10	0 to 6
10^6	10	3 to 7
10^7	10	7 to 9
10^8	10	9 to 10

The range of the numbers of deaths in different experiments with M7 is given in the third column. In each experiment 10 mice received the stated dose.

In smears made from the organs of dead mice, a careful search was necessary to discover any organisms in M7 infection, in contrast with the teeming numbers immediately obvious with M3. A numerical assessment of this difference was made by taking viable counts of organisms recovered from blood, peritoneal and thoracic rinsings, and organs disintegrated by grinding with sand, after dissection of mice immediately after death. With M3, the bacterial population was always of the order of 10^{10} organisms, whereas with M7 the number was most frequently of the order of 10^6 and was never observed to exceed 10^8 organisms per mouse.

The effect on infections with strain M7 of nutrients essential for growth in vitro.

The low population of M7 *in vivo* suggested the possibility of nutritional limitation. Accordingly, substances found necessary for growth of *P. pestis in vitro* at 37° were tested for their effects on M7 infection.

The effect of doses of 4 mg. of each additive (suspended in arachis oil to retard elimination) was tested with a dose of 10^5 M7 bacteria; the results are compiled in Table II.

It was clear that none of the substances tested except haemin affected the lethality of M7. With haemin present, however, 9 or 10 mice per batch of 10

TABLE II.—*Effect of Substances Required* in vitro *when Injected into Mice with M7 Organisms*

Substances.	Deaths per 10 mice.
None	1 to 6
Glucose alone	5
Galactose alone	5
DL-Phenylalanine, L-Cystine, DL-Methionine, DL-Valine, DL-Isoleucine, Glycine, L-Arginine HCl, together	2
Haemin alone	9 to 10

Each substance, whether injected alone or with other substances, was tested at a dose of 4 mg. Single or combined doses, suspended in 0·1 ml. of arachis oil, were injected intraperitoneally with a dose of 10^5 M7 organisms, suspended in 0·1 ml. of buffer.

were killed. Haemin at a level 8 times that used in these experiments was apparently non-toxic to mice in the absence of infection.

Further experiments showed that, under the same conditions, ferrous sulphate, in terms of iron content, was more active than haemin in promoting the lethality of M7 (Table III). Since 4 mg. of $FeSO_4.7H_2O$ was toxic (killing 3 out of 10 mice), the comparisons in Table III were made with smaller doses, which had no apparent effect on uninfected animals.

TABLE III.—*Comparison of Virulence-enhancing Effects of Haemin and Ferrous Sulphate for M7*

	Haemin.		$FeSO_4.7H_2O$.	
Dose (mg.).	Iron content of dose (μg.).	Deaths per 10 mice.	Iron content of dose (μg.).	Deaths per 10 mice.
4·0	340	10	—	—
0·4	34	0	80	10
0·2	17	3	40	10
0·1	9	1	20	10
0·0	0	0	0	0

Dose of M7 organisms = $6·5 \times 10^4$. The doses of haemin and ferrous sulphate used had no apparent effect on normal animals.

It was evident from microscopic examination of smears from the peritoneal exudate and spleen of dead mice that the number of M7 bacteria was enormously increased in the presence of haemin or ferrous sulphate, the numbers seen being then comparable with those observed in M3 infections. This was confirmed by making viable counts of organisms recovered from mice at death. The pattern of virulence also resembled that of M3 in the earlier occurrence of deaths. The enhancement of the peritoneal population of M7 in the presence of ferrous sulphate could be clearly demonstrated in mice sacrificed 40 hr. after infection, as shown in Table IV. At this stage, no deaths were imminent with the ordinary M7 mice,

TABLE IV.—*Effect of Ferrous Sulphate on the Growth of M7 or M3 Organisms in the Mouse Peritoneum*

	Peritoneal population 40 hr. after infection (millions).	
Strain.	Without $FeSO_4.7H_2O$.	With $FeSO_4.7H_2O$.
M7	$7·5 \times 10^{-2}$	643
	$6·1 \times 10^{-3}$	257
	$1·3 \times 10^{-2}$	196
M3	2,675	1,130
	7,050	1,340
	3,700	2,160

The dose of $FeSO_4.7H_2O$ was 0·4 mg., suspended in 0·1 ml. of arachis oil. In the parallel tests without $FeSO_4.7H_2O$, 0·1 ml. of arachis oil was injected. Doses of organisms—M3, $6·0 \times 10^4$; M7, $6·5 \times 10^4$. Three mice of each batch were sacrificed after 40 hr. The peritoneal cavity was rinsed with 1 ml. of citrated buffer, and serial dilutions of this suspension were plated out and counted.

but those which had received iron in addition were all moribund, as were all the M3 mice (the population of M3 did not appear to be influenced by ferrous sulphate).

The results compiled in Table IV suggest that the activity of ferrous sulphate is due to enhancement of the growth of M7 *in vivo* to a level approaching that reached by M3.

Ferric chloride was also active, but salts of other heavy metals, close to iron in the periodic table, were without effect on the virulence of M7 (Table V). The activity appeared to be specific to iron compounds.

TABLE V.—*Effects of Salts of Related Heavy Metals on the Lethality of M7 for Mice*

Salt of heavy metal.	Deaths per 10 mice.
None	3
$MnSO_4.4H_2O$	4
$CoSO_4.5H_2O$	1
$NiCl_2.6H_2O$	2
$CuSO_4.5H_2O$	4
$ZnSO_4.7H_2O$	4
$FeSO_4.7H_2O$	10

Dose of M7 organisms, $1{\cdot}3 \times 10^4$. Dose of heavy metal salt, 0·4 mg. (without apparent effect on uninfected animals).

The virulence of non-pigmented mutants of other strains, and the effects of iron thereon

As previously described (Jackson and Burrows, 1956) the majority of the strains of *P. pestis* tested, after prolonged storage, yielded mixtures of P and NP colonies on pigmentation medium. The NP organisms were presumed to have arisen by mutation from P. Preliminary tests of virulence, using cultures grown from these colonies, suggested that all NP mutants of virulent P strains resembled M7 in having reduced virulence, restored in the presence of iron.

In order to substantiate this result with authentic NP mutants, cultures were prepared from pigmented colonies, and from non-pigmented secondary outgrowths of the same colonies. Mice were infected with these strains in the presence or absence of 0·4 mg. of $FeSO_4.7H_2O$. With avirulent strains and their mutants a dose of 10^7 organisms was used; this was reduced to 10^3 organisms for virulent strains and their mutants. The important vaccine strain, EV76 (Girard and Robic, 1936) was included and also the strain Java. These strains appear to be pure NP. The results are summarised in Table VI.

Notable features of these results are a high correlation between loss of ability to pigment and reduction of virulence in mutants derived from virulent strains: that ferrous sulphate restores the full virulence of such mutants, but does not enhance the virulence of avirulent pigmented strains or of NP mutants derived from them, and that ferrous sulphate renders EV76 fully virulent.

In the case of NP mutants of virulent strains, ferrous sulphate, in addition to restoring full lethality, increased the bacterial population at death, as judged by microscopic examination of smears. The augmented population appeared to resemble that typical of virulent plague infection in all cases except Yokohama NP (*vide infra*).

The exceptional behaviour of L37LP (low pigmentation, but readily distinguished from NP) is discussed below.

TABLE VI.—*Effect of Ferrous Sulphate on the Lethality for Mice of Pigmented Strains and their Non-pigmented Mutants*

	Strain.	Dose.	Deaths per 10 mice. Pigmented strain. Without iron.	Pigmented strain. With iron.	Non-pigmented mutant. Without iron.	Non-pigmented mutant. With iron.
Virulent strains	M3	10^3	10	10	4*	10*
	F9581	10^3	10	10	1	10
	I–72	10^3	10	10	0	10
	139L	10^3	10	10	3	10
	New Mexico 499559	10^3	10	10	1	10
	Shasta	10^3	10	10	2	10
	WCDCPp5A2258	10^3	10	10	2	10
	WCDCPp6	10^3	10	10	0	10
	WCDCPp54	10^3	10	10	0	10
	Yokohama	10^3	10	10	0	10†
	L37	10^3	10	10	5	10
	L37	10^3	10	10	10‡	10
Avirulent strains	A1122	10^7	0	0	0	0
	14	10^7	0	1	1	0
	Soemedang	10^7	0	1	0	0
	Tjiwidej (TS)	10^7	0	0	0	0
Non-pigmented strains	EV76§	10^7	—	—	10	10
		10^3	—	—	0	10
	Java	10^7	—	—	0	1

* This strain is M7.

† Yokohama NP in the presence of iron does not reach such high populations *in vivo* as do other NP strains (see text).

‡ This strain is L37LP (see text).

§ Similar results were obtained with either uracil-dependent or uracil-independent strains (Burrows and Bacon, 1956*b*).

The effect of iron was tested by using a dose of 0·4 mg. $FeSO_4.7H_2O$ in 0·1 ml. of arachis oil.

DISCUSSION

For full virulence it has been established that a strain of *P. pestis* must have all the following capabilities: (1) To elaborate "envelope" antigen (Schütze, 1932), (2) to be highly toxigenic (Englesberg, Chen, Levy, Foster and Meyer, 1954), (3) to develop resistance to phagocytosis in the absence of visible capsulation (Burrows and Bacon, 1956*a*), (4) to synthesise antigens V and W (Burrows and Bacon, 1956*b*), (5) to synthesise purines (Burrows and Bacon, 1954). To this list may be added the ability to produce pigmented colonies on a defined medium containing haemin. This ability appears to reflect a mechanism enabling the fully virulent strain to derive adequate supplies of iron to permit the development of large populations *in vivo*.

It may be tentatively suggested that P strains are able to derive iron from some compound, present in the mouse, which cannot be metabolised by NP strains. The connection between this strain difference and the difference in haemin absorption on a special medium at 28° is at present not understood. We presume that the amount of iron available to M7 in normal mice is always small, but somewhat variable. In some mice, it may be just sufficient to permit

multiplication of the organisms to a lethal level, while in others this level (which is probably much less than the enormous number ultimately reached with highly virulent strains) may be unattainable. This factor, and not the size of the dose, would determine the incidence of deaths, thus affording an explanation of the absence of proportionality between dose and lethal effect with M7. The injection of iron compounds would permit the bacterial population always to exceed the lethal level.

It has proved difficult to make decisive comparisons of iron metabolism between P and NP strains. Experiments with M3 and M7 as representative strains suggest that their growth requirements for iron *in vitro* are quantitatively similar.

The growth of M3 and M7 at 37° in serum from normal mice has been compared, but without significant results, since the bacterial population of both seems to be determined largely by the extent of haemolysis occurring during collection of the serum. Although serum which appeared visually to be free from haemolysis could be collected by very careful methods, no specimen gave a negative benzidine test (King, 1947). Work on this problem is continuing.

A conspicuous anomaly is the behaviour of the mutant L37LP, which, although fully virulent, cannot be assigned to the P group. Further exploration has not, so far, revealed any other strain resembling L37LP.

The appearance of all NP strains is similar after five days' incubation at 28° on pigmentation medium, but further incubation leads in most cases to slight absorption of haemin. This occurs to a different extent with different strains, so that after 10 days most NP strains can be distinguished from each other. L37LP absorbs haemin more strongly than L37NP or any of the others ; at the other end of the scale, Yokohama NP appears to be quite incapable of haemin absorption.

In apparent correlation with these observations, L37LP is evidently not limited by iron deficiency *in vivo*, the population reached without added iron being similar to that with typical virulent strains, while on the other hand, with added iron Yokohama NP does not reach such a high population *in vivo* as other NP strains. (The population reached is sufficient to account for the consistent lethality of Yokohama NP in the presence of iron.)

It is possible that the strain of reduced virulence B741–10–1, isolated from an old laboratory culture by Chen and Meyer (1955) may correspond to the NP type described here.

Attention is drawn to the similarity of the virulence-enhancing effects of iron and of cortisone (Payne *et al.*, 1955) on strain EV76. Both greatly enhance the virulence of this strain, but neither has any apparent effect on the lethality of the avirulent strains TS and A1122.

SUMMARY

M3, a highly virulent strain of *P. pestis*, forms dark brown colonies by absorption of haemin from a suitable medium. M7, a non-pigmented mutant strain cultivated from a non-pigmented secondary colony of M3, has a peculiar pattern of reduced virulence for mice, associated with failure to proliferate freely *in vivo*.

The lethality of M7, and its extent of growth *in vivo*, are augmented to those of M3 if sub-lethal amounts of iron compounds are injected with the organisms. These effects are specific to iron compounds.

In general, non-pigmented mutants of virulent pigmented strains behave like M7 *in vivo*. Non-pigmented mutants of avirulent pigmented strains remain avirulent, even in the presence of iron compounds.

It is postulated that M7 is unable to metabolise some iron compound, present in the mouse, which is utilised by M3. The connection between ability to form pigmented colonies *in vitro* and superior iron metabolism *in vivo* is not fully understood.

It is a pleasure to acknowledge the continued support of Dr. D. W. Henderson and the excellent technical assistance of Mr. B. C. Morris.

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PERIARTERITIS AND OTHER FORMS OF NECROTISING ANGEITIS PRODUCED BY VITAMIN D IN THYROXINISED RATS WITH AN ASSESSMENT OF THE AETIOLOGY OF THOSE VASCULAR LESIONS

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THE results to be reported in this study arose from an enquiry into the factors regulating the structural integrity of the cardiovascular system (Gillman, 1955 : Gillman and Gilbert, 1956*a*, *b*). As part of this enquiry, it became necessary to examine more closely the manner in which vitamin D injured the arteries of the body.

While it is generally accepted that vitamin D intoxication can lead to extensive deposition of calcium in the arterial system (Reed, Struck and Steck, 1939), and can cause temporary interference with kidney function (Anning, Dawson, Dolby and Ingram, 1948), necrotising arteritis, of the kind described in periarteritis nodosa, appearing during the acute stages of vitamin D-intoxication has hitherto escaped attention. In a brief report, Ham (1944) mentioned the development of periarteritis in the intestinal arteries and extensive hyalinisation of the glomeruli of the kidneys of rats six months after feeding either a single massive dose of activated ergosterol or a diet rich in calcium and phosphorus for three weeks. In our experiments, arteritis was detected in some of the rats within two days after the cessation of a 5-day course of treatment with massive doses of vitamin D. This finding led to a search for those conditions which could promote the consistent development of periarteritis in rats receiving excess vitamin D.

It will be the purpose of this study, first to demonstrate that periarteritis can be produced in rats receiving vitamin D and that such vascular lesions are not necessarily associated with extensive kidney changes, secondly to describe the pathogenesis and repair of the lesions observed and thirdly, to examine the relevance of the findings for the understanding of the various forms of periarteritis described in man.

From an examination of the available data in both animals and man, it will be suggested that derangement of a particular function of the kidney, perhaps affecting directly or indirectly the metabolism of calcium and phosphorus, plays a central part in the production of arteritis. Such a derangement in function may not necessarily be accompanied by extensive structural change in the kidney.

Finally, in view of the possible significance of this observation for the understanding of the frequently reported chronic intoxication in infants receiving therapeutic amounts of this vitamin (Schlesinger, Butler and Black, 1956) the importance of the endocrine glands in modifying the sensitivity of rats to vitamin D will be stressed.

MATERIALS AND METHODS

Five series of rats were used for this investigation. The first, consisting of 220 males and 80 females, received 100,000 units vitamin D/kg. body weight daily for 5 consecutive days and were killed at various intervals thereafter. The second series, consisting of 52 male rats, received pre-treatment with thyroxin (40 μg. thrice weekly) for 21 days and thereafter, 42 of these animals received the same course of vitamin D as the first series. The remaining 10 animals served as controls. Thyroxin was continued at the same intervals during treatment with vitamin D and after the vitamin was discontinued until the rats were killed. The third series, consisting of 21 male rats, was given pre-treatment with cortisone (1 mg. daily) for 21 days, followed by a course of vitamin D treatment. Cortisone injections were continued throughout the experiment and until the rats were killed. An additional 10 rats received cortisone injections only and served as controls to the vitamin D-treated rats. The fourth series composed of 20 male rats received pre-treatment with desoxycorticosterone acetate (doca, 1 mg. daily) for 7 to 14 days before a 5-day course of treatment with vitamin D. Ten control rats received injections of doca only. As in the second and third series, doca injections were maintained during as well as after vitamin D administration until the rats were killed. The fifth series of 10 male rats were totally thyroidectomised. One month after thyroidectomy, a 5-day course of treatment with vitamin D was given and the animals were killed 2–8 days after the last dose.

All the animals weighed 230–270 g. and were fed the stock ration of our colony (Gillman, Gilbert and Spence, 1953). By chemical analysis, the calcium content of the diet was found to be 0·36 and the phosphorus content 0·38 mg. per 100 g., giving a calcium and phosphorus ratio of 1 : 1.

Vitamin D, in the form of crystallised calciferol, was dissolved in arachis oil and fed to the rats by dropper.

RESULTS.

Types of Lesion Produced

At least six varieties of reactions may be encountered in the arteries and veins of rats killed 48 hours after a 5-day course of treatment with vitamin D, namely, (i) oedema and colliquative necrosis of the intima and media with or without haemorrhage, periarterial oedema or periarteritis; (ii) periarteritis affecting small arteries, accompanied by a variable degree of endarteritis, medial damage and an intense concentration of eosinophile polymorphs in the lumen, wall or around the vessel; (iii) necrotising arteritis affecting a segment or the entire circumference of the artery, associated with fibrinoid degeneration and intense periarteritis which may acquire all the features of a granuloma; (iv) necrotising arteritis with or without haemorrhage but with minimal response of the tissues around the artery; (v) necrotising arteritis involving the intima and media, followed later by an intense endothelial and subendothelial cellular hyperplasia; (vi) endophlebitis, periphlebitis or even panphlebitis with or without giant cell formation. All these reactions may be encountered in one and the same animal although on occasion one reaction may predominate over the others.

Reaction 1.—Periarterial and intramural oedema of the artery commonly affects the extrarenal segment of the renal artery usually extending from its origin and terminating just before entering the hilum of the kidney. In the early stages, although the endothelial lining is still intact, the muscle cells are greatly swollen, small vacuoles lie within and between the muscle fibres and the internal and external elastic laminae are accentuated (Fig. 1). Intense oedema of the adventitia renders this layer no longer distinguishable from the surrounding oedematous connective tissue.

The early reaction may be followed rapidly by karyorrhexis and even desqua-

mation of the endothelial cells. The cellular elements included between the two elastic laminae liquefy with the result that only a few degenerated nuclei are to be found strewn irregularly through the débris (Fig. 2). Furthermore, the internal elastic lamina may also undergo dissolution in some regions. Periarterial oedema is intense but there is little, if any, proliferation of the connective tissue cells in the adventitia.

At a later stage, the granular contents of the media may become eosinophilic. Dissolution of a localised segment of the internal elastic lamina results in great weakening of the wall and may be followed by haemorrhage into the media. However, as a rule the blood is contained within the media by the persisting external elastic lamina, rupture of which would certainly result in severe local haemorrhage (Fig. 3).

In some circumstances not yet understood, liquefaction of the media may be followed by rapid dehydration of the contents with the formation of a hyaline tissue which replaces the entire wall of the artery (Fig. 4). Although the oedema may persist in the adventitia, the connective tissue cells may be enlarged and more numerous than usual. As will be shown later, repair of such an injury is effected mainly by ingrowth of the cells from the adventitia and from the unaffected parts of the media.

On the other hand, colliquative necrosis may be confined to a small part of the media (Fig. 5). In such instances, the muscle cells are separated by the accumulation of fluid and pycnosis of the nuclei of adjacent cells often occurs together with injury to the overlying intima. A mild periarteritis extends for some distance on either side of the lesion. Even where the injury is restricted, disruption of the related segment of the internal elastic lamina and endothelium results in a small intramural haemorrhage which accentuates the initial damage (Fig. 6). These small intramural haemorrhages are usually accompanied by some periarteritis which may be intense.

Reaction 2.—This reaction is characterised by a mild periarteritis and endarteritis and by the accumulation of eosinophile polymorphs in and around the wall of the artery (Fig. 7, 8). The small muscular arteries are mainly affected. In such vessels, the endothelial cells enlarge, proliferate and give rise to small syncytial masses projecting into the lumen of the arteriole (Fig. 8). The media does not appear to be obviously damaged. However, the cellular reaction may be intense in the adventitia, extending irregularly into the surrounding connective tissue (Fig. 9).

In the arterioles, the swollen endothelial cells and the large number of eosinophiles may almost plug the lumen (Fig. 7) ; the muscle cells degenerate and the media as well as the surrounding adventitia may be crowded with eosinophiles. The vessels may be completely destroyed leaving a circumscribed area of necrotic débris infiltrated by degenerating eosinophiles and proliferating connective tissue cells. Such reactions usually occur at the sites of bifurcation of the small muscular arteries (Fig. 9) although they may be found affecting a segment of an artery where no obvious branch can be found in the neighbourhood.

Reaction 3.—This may be regarded as classical periarteritis nodosa described in rats by the majority of investigators after injuring the kidneys by one or more of a variety of techniques. As repeatedly emphasised by Zeek, Smith and Weeter (1948) and Smith, Zeek and McGuire (1944) and Smith and Zeek (1947), such lesions are localised to the site of bifurcation of arteries. However, peri-

arteritis nodosa is not restricted to medium-sized arteries but may occur even in arterioles lying in the submucosa of the stomach, caecum, large bowel as well as in the large renal, mesenteric, and pancreatic arteries.

The grades of intensity of the reaction observed are shown in Fig. 10, 11, 12. In its mildest form, the inflammatory reaction is confined to a small zone in and immediately beyond the adventitia while the wall itself may be injured only in one segment; sometimes the inflammatory reaction appears to be out of proportion to the degree of injury to the vessel itself. Furthermore, a granuloma may form some distance beyond the artery (Fig. 12, 13).

As a rule, the wall of the artery shows a characteristic necrosis of the media, immediately deep to and often implicating the intima and even the endothelium (Fig. 11, 12). On occasion the entire wall may be replaced by fibrinoid material and giant cells may develop in the intima (Fig. 11). The pleomorphic cellular reaction consisting of fibroblasts, histiocytes, monocytes and polymorphs is seen in Fig. 13. Indeed, the periarteritic lesions described above are indistinguishable from those produced by Bali and Goldblatt (1954) following constriction of the renal artery of one kidney and removal of the other.

Reaction 4.—The ostia of the coronary arteries and the epicardial segment of the vessel are particularly vulnerable to the effects of vitamin D. Subendothelial oedema and necrosis of the adjacent media occur frequently, to be followed by the accumulation of monocytes and polymorphs below the endothelium. The latter in turn may proliferate to add to the cellularity of the intima. During the stage of repair, many giant cells may be found in the intima. This lesion is very similar to the one described by Hueper (1942, 1944) in dogs treated with various macromolecular substances.

Reaction 5.—Endophlebitis, periphlebitis and panphlebitis often occur in association with one or other variety of arteritis described above. Both medium-sized and small veins are affected, including those of the stomach and large gut and especially those of the submucosa of the caecum and the larger veins draining this viscus. The veins alone may be involved, without any detectable lesion in the adjacent arteries (Fig. 14), or one or more layers of the vein may be injured while a closely related artery may show intense periarteritis (Fig. 15) which has not necessarily spread directly to the vein. The possibility cannot be discounted that some metabolite which has stimulated the periarteritis in the first instance has also affected the related vein.

Both the endothelium and the adventitia are usually simultaneously implicated (Fig. 14, 15, 16). Proliferation of the endothelium with the formation of giant cells is frequent (Fig. 16). These giant cells are derived from endothelium of the smaller venules and capillaries which drain the wall of the larger vessel. In this connection it should be mentioned that some of the smaller veins receive as tributaries numerous thin-walled venules, concentrated over a small segment of the vein. In ordinary circumstances, the blood courses freely from the tributary through the walls into the lumen of the large vein. Contraction of the thin layer of smooth muscle immediately deep to the endothelium of the receiving vein may prevent drainage of blood from the delicate tributaries. The dilated tributaries then form a complex maze of blood-filled channels in the wall of the receiving vein. Indeed, periphlebitis usually begins near a confluence of venules with a larger vein.

Despite severe injury to the veins, thrombosis is rarely encountered.

Endocrine Factors Modifying the Vascular Lesions

These reactions did not occur with equal consistency in all rats receiving the specified treatment with vitamin D nor were all arteries and arterioles equally affected. The extra-hilar segment of the renal artery is most frequently injured and thereafter, with decreasing frequency, the coronary, caecal, gastric, superior mesenteric and splenic arteries. Reaction 1, usually unaccompanied by intramural haemorrhage, was observed in the renal artery, reaction 4 without haemorrhage in the coronary and mesenteric artery and, occasionally, reactions 3 and 6 in the caecal artery and veins respectively.

Rats receiving thyroxin before, during and after treatment with vitamin D show a marked intensification of the arterial lesions, few if any rats escaping vascular damage. The coronary arterial injury is manifest as necrosis of the intima and media followed later by proliferation of the endothelium (reaction 5), almost always accompanied by widespread necrosis of the ventricular myocardium. Reactions 1 and 3, the former with haemorrhage, are to be found in the renal artery as well as in the gastric and mesenteric arteries. Massive necrosis and fibrinoid degeneration, with the formation of a granuloma, occur with almost unfailing regularity in the pancreatic and splenic arteries. Reaction 6 is commonly observed in the caecal veins and reactions 2, 3 or 5 in the caecal arteries. Furthermore, it is noteworthy that haemorrhagic necrosis of the spleen occurred in 21 per cent of these animals in contrast to 2 per cent in rats not receiving thyroxin (Gillman and Gilbert, 1956*b*).

Treatment with doca, before, during and after the administration of vitamin D emphasised the haemorrhagic and necrotising lesions, particularly in the large arteries, such as the renal, superior mesenteric and epicardial segment of the coronary. Indeed, the severity of reaction 1 is consistently increased in rats receiving doca.

Unlike thyroxin and doca, cortisone offered a considerable measure of protection against the lesions induced by vitamin D. At most, the medium-sized arteries, including the renal, usually very vulnerable to vitamin D, showed slight hyalinisation of the wall with early oedema and occasionally early periarteritis. Neither reaction 2 nor reaction 6 was observed in any of the cortisone-treated rats.

Removal of the thyroid gland a month before treatment of the rats with vitamin D greatly reduced the arterial damage. The renal arteries were normal in four rats, and in the remaining six the injury could be classified at most as a mild reaction 1. None of the rats showed either reaction 2 or 6.

It was not possible with any of the procedures used to protect the rats completely from the vasculo-toxic effects of vitamin D. Nevertheless thyroidectomy and the administration of cortisone markedly decreased the intensity of the vascular lesions while doca, and particularly thyroxin, aggravated them.

Repair of Vascular Lesions

The manner in which repair is achieved and the sequelae of arteritis depend on the nature and extent of the initial injury. Oedema of the wall of the adventitia may resolve completely. On the other hand, repair of severe arteritis (Fig. 2) is a much more protracted affair. The periarterial oedema subsides, water is

resorbed from the necrotic media which in turn is converted into a dense hyaline mass, separated from the lumen by an irregular layer of abnormal endothelial cells. At one stage, the artery may resemble that shown in Fig. 4 and that in Fig. 17 at a much later stage. Capillary ingrowths from the adventitia and the migration of fibroblasts and histiocytes participate in repairing the damaged wall. The hyaline material is removed and may persist indefinitely as a layer deep to the endothelium (Fig. 18). In some instances a localised scar and a bizarre arrangement of the cells (metallaxis) may disclose the final stage of repair.

Acute necrosis of a segment of an artery stimulates both an endothelial and an adventitial response (Fig. 19). Cells invade the necrotic media from both directions with the result that the hyaline layer becomes wedged between two cellular layers. As mentioned in the case of the larger branches of the coronary arteries, intramural capillaries may be formed from the endothelial cells of the intima and by ingrowth from the adventitia (Gillman and Gilbert, 1956*a*). As the endothelial lining is restored, the intima is vascularised and contains detached endothelial cells (Fig. 20). For a while, the wall as a whole is greatly thickened and consists of dilated capillaries and mobilised endothelial cells which elaborate an abundant matrix (Fig. 20). Later, with occlusion of the capillaries and the elaboration of collagen deep to the intima, in which a few giant cells occasionally develop from capillary endothelium, repair again results in a scar.

Restitution or complete occlusion with disruption of the vessel wall by fibrous tissue, which is surrounded by eosinophiles, plasma cells, histiocytes and a few lymphocytes, may be the fate of such lesions as those shown in Fig. 7 and 8.

Repair of the vitamin D + thyroxin-induced periarteritis is scarcely different from that of arteritis produced by one of the several modifications of the Goldblatt technique. Several stages in repair are shown in Fig. 10, 11 and 21. Thrombosis may complicate and delay repair (Fig. 11). The original media is usually replaced by a hyaline band and a few giant cells may be derived from the endothelium. Collagen is elaborated in the adventitia. Eventually, the wall of the vessel is greatly thickened and a band of hyaline material persists deep to the endothelium.

DISCUSSION

From the foregoing, it is evident that vitamin D is capable of producing a wide range of vascular reactions in rats. Some of these reactions conform to the classical descriptions of periarteritis nodosa and to the hypersensitivity angeitis and rheumatic arteritis described by Zeek (1952, 1953). This author believed that each of these forms of arteritis was a distinct morphological entity, having its own particular aetiology. Since, in our experience, these several vascular reactions could be observed in one and the same animal, they can be regarded as manifestations of a disturbance of a common underlying process. Such a view is in accord with that expressed by several investigators, namely, that the rheumatic and pararheumatic diseases have many pathological features in common (Roesslé, 1936 ; Masugi and Yae-Shu, 1938 : Klemperer, Pollack and Baehr, 1941, 1942 ; Fox, 1943), and furthermore, that many similarities exist between these spontaneously-occurring conditions in man and those produced in animals by numerous seemingly unrelated techniques (Vaubel, 1932 ; Klinge, 1933 ; Knepper and Waaler, 1934–35 ; Rich and Gregory, 1943 ; McKeown, 1947 ; More and McClean,1949 ; Ehrich, Seifter and Forman, 1949). However, the remarkable

divergences in response to a defined stimulus even in the same animal still require explanation.

The kind of lesion which develops in response to vitamin D depends in part on the amount administered as well as on the endocrine state of the animal. The kind of lesion observed in an artery will also depend on the time at which it is examined after application of the stimulus. All the acute lesions, including eosinophilic and fibrinoid degeneration of the wall of the artery, haemorrhage, perivascular oedema and mild forms of periarteritis and periphlebitis are usually encountered within 2 days after the last of a series of 5 doses of vitamin D. Intense inflammatory processes declare themselves 3 to 4 days later and the sclerosing lesion is rarely observed before the 8th to the 15th day. At this time, the mild forms of injury are almost healed; metallaxis may be the only evidence of previous damage.

Quite apart from the endocrine condition and the amount of vitamin D administered, regional differences in tissue metabolism and locally operating mechanical factors may also influence the nature of the reaction. Thus, even in thyroxinised vitamin D-treated rats, the intrapulmonary arteries and veins almost invariably escape injury. Furthermore, Salgado and Selye (1955) have shown that overdosage with methylandrostenediol in rats produces a syndrome consisting of nephrosclerosis, myocarditis, periarteritis nodosa, hypertension and polyuria; this syndrome failed to develop in the absence of the adrenals.

In rabbits, the inconsistent and variable response in the vascular system following the injection of horse serum and some of the fractions thereof has been attributed to such factors as the speed of administration of the serum (Orbison, 1952), the amount of serum injected at a time (McClean, Fitzgerald, Younghusband and Hamilton, 1951) and even the quality of the serum (Hawn and Janeway, 1947). Indeed, it may be said that the quality and intensity of the stimulus, taken in conjunction with the state of reactivity of the organism can modify profoundly the response elicited. Where the quality and intensity of the stimulus remain constant, divergent responses reflect differences in the physiological state of the organism.

Aetiological considerations

The aetiology of periarteritis nodosa and of the other forms of necrotising angeitis has been reviewed by Grant (1939–42), Wilson and Alexander (1945), Urbach and Gottlieb (1946), More and McClean (1949), Zeek (1952, 1953), Goldblatt (1950), Bali and Goldblatt (1954) and many others. It would appear from the literature that hypersensitivity, kidney disease and hypertension play a part in the aetiology of the various forms of angeitis in man and animals alike.

Hypersensitivity.—The importance of hypersensitivity has been emphasised ever since Gruber (1925*a*, *b*) suggested that periarteritis nodosa was caused by cocci in the presence of an underlying allergy. The frequent occurrence of periarteritis in individuals with a history of allergy added credence to Gruber's original viewpoint (Urbach and Gottlieb, 1946). Moreover the production experimentally of necrotising angeitis in rabbits by intravenous injection of *Bact. coli* (Masugi and Isibasi, 1936) by injection of horse serum (Rich and Gregory, 1943), and foreign proteins of bacterial and non-bacterial origin (Klinge and Vaubel, 1931; Metz, 1932; Vaubel, 1932; Roesslé, 1933) seemed to provide further confirmation

of the importance of hypersensitivity as a factor promoting necrotising angeitis, especially as antibodies were identified in rabbits developing arteritis after the injection of the foreign protein.

The fact that hypersensitivity to a foreign protein can be induced in the rabbit does not necessarily imply that the state of hypersensitivity is causally related to the ensuing arteritis. Indeed, Ehrich (1947) was of the opinion that the vascular injury might be the direct result of large amounts of injected horse serum rather than the result of the subsequently developing state of hypersensitivity. In this connection, it should be mentioned that the various techniques which have been used for the production of so-called hypersensitivity arteritis in rabbits almost invariably induced nephritis of greater or lesser severity; the nephritis in such experiments has been regarded as a manifestation of hypersensitivity. It is more than likely that horse serum directly injures the kidney which, as will be seen later, may in itself promote vascular disease in particular circumstances.

Little support for hypersensitivity as a factor in the causation of arteritis is forthcoming from experiments in rats. It is unlikely that the arteritis evoked in vitamin D-treated thyroxinised rats can be ascribed to hypersensitivity, especially as the rat is known to be particularly resistant to anaphylaxis or other similar antigen-antibody responses. On this basis, it can be presumed either that the mechanism underlying arteritis in rabbits injected with foreign proteins is different from that induced in rats by a variety of techniques or that disturbances of a common mechanism result in the development of arteritis in both species.

Kidney disease.—The injection of foreign proteins into rabbits is frequently followed by the development of gross pathological changes in the kidney. In their original experiment, Rich and Gregory (1943) reported glomerulonephritis in 10 of 14 rabbits injected with horse serum. Partial or complete destruction of the kidney by a variety of techniques is also followed by the development of various kinds of arteritis in a large percentage of rats (Goldblatt, Lynch, Hanzal and Somerville, 1934; Friedman, Jarman and Klemperer, 1941; Kempf and Page, 1942; Cromartie, 1943; Smith, Zeek and McGuire, 1944; Loomis, 1946; Goldblatt, 1948; Koletsky, 1953; Masson, Del Greco, Corcoran and Page, 1953*a*; Masson, Plahl, Corcoran and Page, 1953*b*; Masson, Fisher, Corcoran and Page, 1955; Bali and Goldblatt, 1954). In our experiments, necrotising lesions of the artery were consistently associated with variable degrees of kidney change. In dogs, bilateral nephrectomy or direct injury to the kidney also results in arteritis (Grollman, 1950). In man, Davson, Ball and Platt (1948) and Wainwright and Davson (1950) have reported rapidly developing glomerulitis in some of their cases of periarteritis nodosa and gross renal disease has been repeatedly emphasised (Gruber, 1925*a*, *b*; Gray, 1929; Arkin, 1930; Harris, Lynch and O'Hare, 1939). It would appear therefore that the co-existence of periarteritis nodosa and kidney disease is not entirely fortuitous.

It is clear from the observations of Davson *et al.* (1948) that severe periarteritis nodosa may exist without corresponding gross involvement of the kidney. Furthermore not all kidney injury will automatically result in vascular disease. We have failed to detect arteritis in nutritionally produced chronic nephritis in rats or in vitamin A-deficient rats which had developed multiple renal abscesses or pyelonephritis. Acute or chronic kidney disease, therefore, does not automatically presuppose the co-existence of periarteritis nodosa although this may supervene in individuals already suffering from kidney disease.

EXPLANATION OF PLATES

All rats received a 5-day course of treatment with vitamin D (100,000 units/kg. body weight) and were killed 2–15 days thereafter.

FIG. 1.—Early degeneration of wall of renal artery with pycnosis of the nuclei in the media, prominence of the endothelium and intense periarterial oedema. Reaction 1: 2 days after last dose vitamin D; rat receiving thyroxin treatment. × 450.

FIG. 2.—Liquefied cells of the media enclosed between the internal and external elastic laminae, localised dissolution and "blurring" of the internal elastic lamina and periarterial oedema in the renal artery. Reaction 1: 2 days after last dose vitamin D; rat receiving thyroxin treatment. × 450.

FIG. 3.—Localised haemorrhage into the media of a caecal artery with oedema and early periarteritis. Reaction 1: 2 days after last dose vitamin D; rat receiving thyroxin treatment. × 450.

FIG. 4.—Homogenisation of the intima and part of the media of caecal artery with resultant formation of hyaline material replacing almost the entire arterial wall. Note oedema of the adventitia and enlargement of the connective tissue cells. Reaction 1: 4 days after last dose vitamin D; rat receiving thyroxin treatment. ×450.

FIG. 5.—Longitudinal section of gastric artery showing swollen endothelial cells, oedema and vacuolation of muscle cells and mild adventitial oedema. Reaction 1: 2 days after last dose vitamin D; rat receiving thyroxin treatment. × 450.

FIG. 6.—Extravasation of red cells in the wall of the gastric artery; swelling of muscle cells and endothelium with periarteritis and adventitial oedema. Reaction 1: 2 days after last dose vitamin D. × 450.

FIG. 7.—Arteriole of the caecum showing swollen endothelial cells, abundant eosinophilic polymorphs almost occluding the lumen and crowding the adventitia. Reaction 2: 2 days after last dose vitamin D; rat receiving thyroxin treatment. × 450.

FIG. 8.—High power of small muscular artery in the caecum shown in Fig. 9, with endothelial cells forming a syncytium in the lumen. Intense cellular reaction in the adventitia. Reaction 2: 2 days after last dose vitamin D; rat receiving thyroxin treatment. × 450.

FIG. 9.—Mild periarteritis and endarteritis associated with polymorph invasion of the adventitia and localised endothelial proliferation at site of branching of artery in the caecum. Reaction 2: 2 days after last dose vitamin D; rat receiving thyroxin treatment. × 100.

FIG. 10.—Panvasculitis with necrosis of the intima and media of a caecal artery. Reaction 3: 2 days after last dose vitamin D; rat receiving thyroxin treatment. ×100.

FIG. 11.—Hyalinisation of the intima and related media with giant cell formation in the endothelium and resolving periarteritis in caecal artery. Reaction 3: 4 days after last dose vitamin D; rat receiving thyroxin treatment. × 100.

FIG. 12.—Intense periarteritis at site of branching of a gastric artery with focal necrosis of intima and media of both vessels and formation of granuloma in the adventitia and related connective tissue. Reaction 3: 2 days after last dose vitamin D. × 100.

FIG. 13.—High power of granuloma in Fig 12 showing pleomorphic cell pattern. × 450.

FIG. 14.—Panphlebitis with formation of giant cells in intima in vein of caecum. Related arterioles are unaffected. Reaction 5: 2 days after last dose vitamin D; rat receiving thyroxin treatment. × 100.

FIG. 15.—Periarteritis and localised endo- and peri-phlebitis in caecal vessels. Reaction 5: 2 days after last dose vitamin D; rat receiving thyroxin treatment. × 100.

FIG. 16.—High power of caecal vein in Fig. 14 showing giant cells in intima, enlarged endothelial cells and mild periphlebitis. × 450.

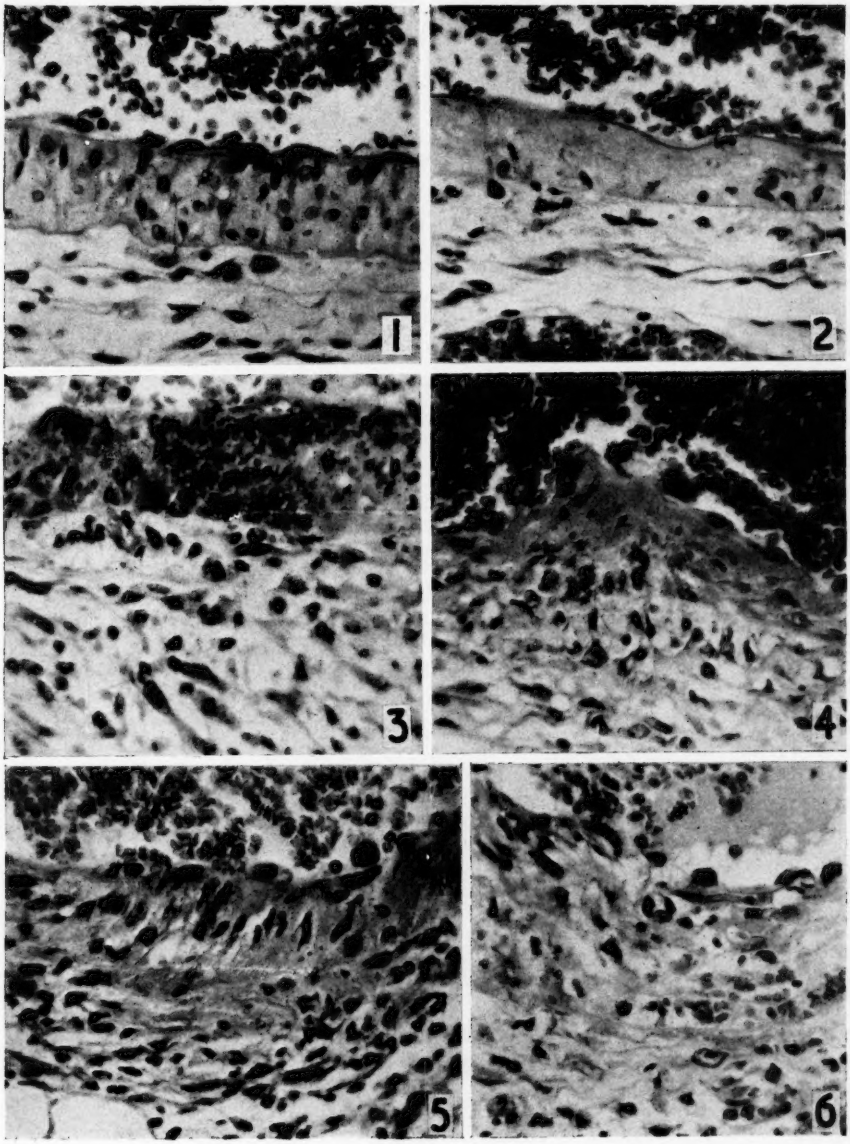
FIG. 17.—Reduced periarteritis, metallaxis and absence of sharp distinction between media and adventitia in caecal artery. Healing lesion: 9 days after last dose vitamin D; rat receiving thyroxin treatment. × 450.

FIG. 18.—Mild hyalinisation of subintimal tissues with metallaxis in caecal artery. Note increased cellularity in adventitia and loss of oedema. Healing lesion: 9 days after last dose vitamin D; rat receiving thyroxin treatment. × 450.

FIG. 19.—Repair of localised necrosis of intima, proliferation of endothelial cells with detachment and rounding off of an endothelial cell in pancreatic artery. Note early hyalinisation in the media and residual periarteritis. Healing lesion: 8 days after last dose vitamin D; rat receiving thyroxin treatment. × 450.

FIG. 20.—Cellular thickening of the intima with dilated capillaries and resolving periarteritis of the pancreatic artery. Eight days after last dose vitamin D; rat receiving thyroxin treatment. × 450.

FIG. 21.—Healing periarteritis with residual necrosis in part of the media and early hyalinisation of the pancreatic artery. Fifteen days after last dose vitamin D; rat receiving thyroxin treatment. × 100.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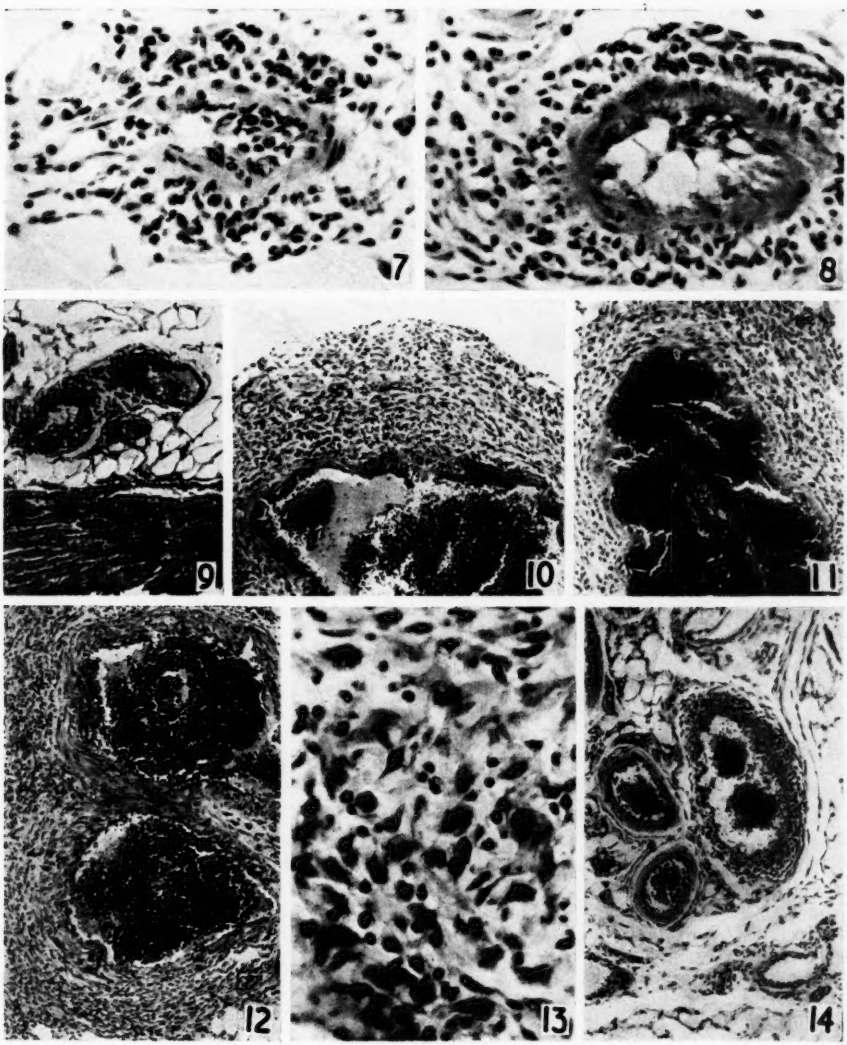
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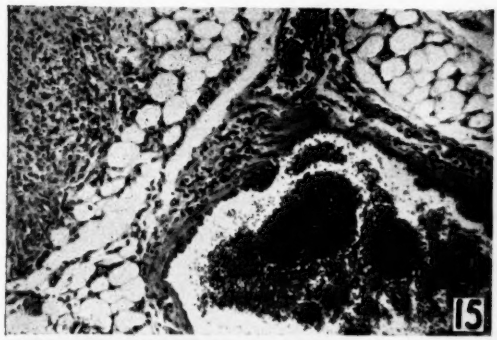
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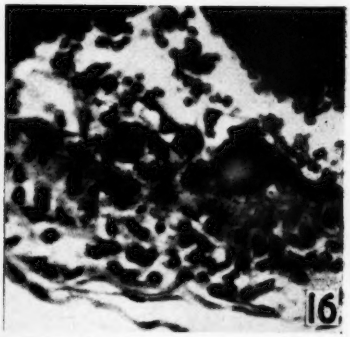
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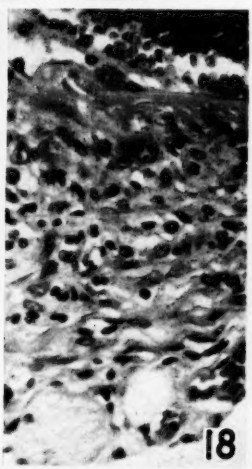
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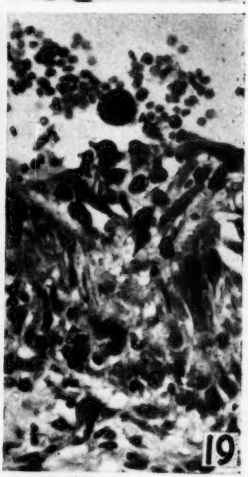
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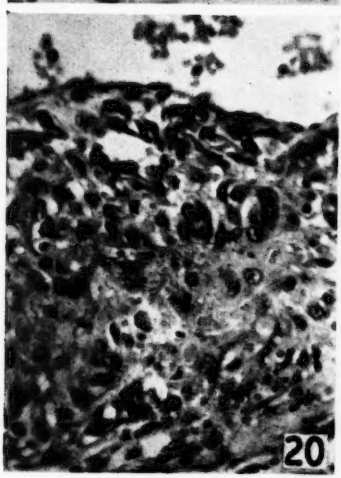
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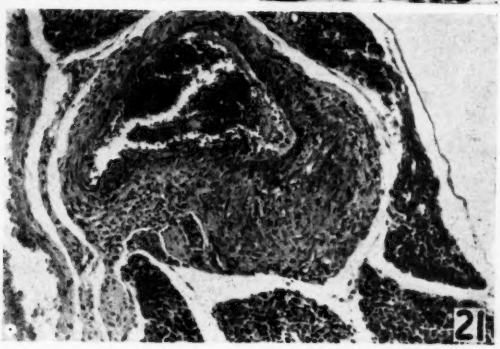
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On the other hand, periarteritis may develop following functional derangement of the kidney. Thus, Cromartie (1943) induced a perinephritis attended by widespread arteritis without structural alterations in the kidney itself. Transitory injury to the kidney may be attended by a single episode of acutely developing vascular lesions. In our experiments necrotising arteritis declared itself 8 days at the most after the first treatment with vitamin D. Such lesions may heal slowly and residual injury to the vessel wall may still be detected several months later. When the kidney lesion is progressive or where there have been numerous acute episodes, followed by remissions, all stages in the development and the repair of the vascular lesions may be encountered in the same animal. After an acute episode, the kidney lesion may recover almost completely. As a consequence, there may be no obvious correlation between the extent of kidney changes and the gravity of the vascular lesion.

Progressive kidney disease, unless accompanied by a particular disorder in renal function, is not attended by periarteritis. Hence, it is possible to envisage the following: (1) Mild kidney disease with extensive periarteritis, (2) severe kidney disease without arteritis, (3) severe kidney disease with severe arteritis, (4) severe kidney disease on which may be superimposed that specific modification in function which will promote an acute arteritis. In the latter instance, the arteritis may be a terminal event or may develop very late in the evolution of chronic nephritis.

Hypertension.—The frequently developing hypertension following injury to the kidney has been said to strain excessively the vascular system which eventually breaks down in the form of arterial necrosis (Byrom and Dodson, 1948; Pickering, 1952). Within this context, Grollman (1950) believed that infection was an important aetiological factor, a view which was rejected by Goldblatt (1950) and Page (1950). The association of pulmonary hypertension and periarteritis in the lungs has emphasised the importance of a raised blood pressure in the aetiology of arteritis in general (Symmers, 1952).

It is unlikely that an elevated blood pressure *per se* will lead necessarily to periarteritis nodosa, a view previously expressed by Goldblatt (1950). In the first place, arterial lesions are infrequent in experimental neurogenic hypertension (Goldblatt, 1950). Secondly, following injury to the kidney, 43 per cent of animals suffering from hypertension, as well as uraemia, did not develop necrotising arteriolar lesions (Bali and Goldblatt, 1954). Thirdly, in our small series of 20 animals in which blood pressures were measured, necrotising lesions appeared without any detectable elevation in blood pressure. Moreover there was no histological evidence of ante-mortem arteriolar constriction; indeed, the diameter of the lumen of the vessel which suffered injury was rarely, if ever, reduced (Fig. 8, 10, 12, 15, 21). It would seem therefore that hypertension plays a secondary rôle in the aetiology of arteritis.

How the kidney regulates the integrity of the cardiovascular system still remains to be established. When kidney damage first declares itself in vitamin D-treated rats, mineralisation of the tissues can already be detected chemically but not histologically, even though the serum calcium and phosphorus are either unchanged or at most at the upper limits of normality. Moreover, mineralisation of the tissues implies changed tissue reactivity. The close time relationship between the appearance of kidney lesions and mineralisation of the tissues at the chemical level does not permit us to state at this stage whether vitamin D acts

primarily on the kidney, primarily on the tissues or simultaneously on both. Whatever the sequence, once the tissue reactivity is altered, calcium accumulates in greater or lesser quantities in the large arteries.

If such calcium accumulates in an ionic form, tissue damage inevitably ensues. Since considerable quantities of calcium can be deposited in the vessel without obvious damage, it can be inferred that the calcium in the tissues exists in a bound form. Excessive binding of calcium may decrease the locally available calcium ions. Such a decrease could easily lead to relative potassium excess with loss of tone in the affected artery directly or, possibly, indirectly by reducing the creatine phosphate which, according to Born (1956), is apparently necessary for the maintenance of tone in smooth muscle. This loss of tone taken in conjunction even with a normal blood pressure, may be responsible for dilating the affected arteries and arterioles before they undergo necrosis.

Excessive elevation of the blood pressure may intensify further the vascular injury but does not in itself apparently promote vascular injury. It is significant that in all cases of spontaneously occurring arteritis in man, as well as in experimental animals, the affected vessels are dilated and only become narrowed when the lesion is chronic, due to the inflammatory processes.

The pathogenesis of vitamin D-produced arteritis seems to occur in at least two stages, namely, changed tissue reactivity which, in turn, promotes mineralisation and damage to the vessel wall. However, since acute arteritis developing in vitamin D-treated animals is not obviously different from that resulting from direct tampering with the kidney in rats, it would seem that disturbance of the particular function of the kidney may be coupled in some way with a modification in calcium and phosphorus metabolism which may not necessarily be detected by any significant change in these ions in the circulating blood. This possibility is still further strengthened by the repeated association of particular forms of kidney injury with diffuse arterial calcification, involving small and large arteries alike. Whether or not the kidney disorder will be accompanied by vascular lesions may then depend on some change in the metabolism of calcium and phosphorus and the changed affinity of the tissues for these ions. Severe disturbances in calcium and phosphorus metabolism may result in extensive calcification of the vascular tree, while less severe disturbances may result in necrotising arteritis and in the other vascular lesions described above.

Finally, whether arteritis is produced by vitamin D or any other experimental technique, it is evident that at least the endocrine configuration of the organism will influence the nature and the intensity of the resulting lesions. This observation may be of importance in explaining the increased susceptibility of infants and children to the toxic effects of seemingly physiological concentrations of vitamin D, and may account, perhaps, for some of the cases of idiopathic hypercalcaemia described in children (Schlesinger *et al.*, 1956; Creery, 1953; Anning *et al.*, 1948). The experiments reported in our rats strongly suggest that an active thyroid gland in particular potentiates the calcifying effects of vitamin D and, in addition, promotes a high incidence of periarteritis and necrotising arteritis.

SUMMARY

Various kinds of acute necrotising angeitis have been produced in rats receiving vitamin D. The lesions develop rapidly, appearing within two days after a five-day course of treatment.

The lesions were greatly intensified and appeared more consistently in rats which received thyroxin before, during and after the course of treatment with vitamin D. The simultaneous administration of desoxycorticosterone acetate tends to promote the haemorrhagic form of arteritis while cortisone and thyroidectomy offer a considerable measure of protection.

If the treatment with vitamin D was stopped, the arterial lesions were shown to be transitory and repair could be effected within 17 days, although residual injury could be detected in the vessels many months later.

Some forms of the vitamin D-produced arteritis simulated those produced in rabbits following massive injections of horse serum while others resembled those occurring in rats after drastic interference with the kidney. The arterial lesions in the vitamin D-treated rats were also similar to those described in man, developing spontaneously in rheumatic and pararheumatic diseases.

It is suggested that generalised arteritis in man, as well as that produced experimentally in animals, can be related to a common underlying mechanism, dependent on derangement of a particular function of the kidney, but not necessarily dependent on gross structural disorganisation of the kidney or on any change in the blood pressure. It is further suggested that the kidney disorder favouring arteritis is coupled in some way to the altered metabolism of calcium and phosphorus and to the changed affinity of the vascular tissue for these ions.

Finally, attention is drawn to some of the endocrine factors which could modify the sensitivity of experimental animals to vitamin D intoxication.

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THE INFLUENCE OF A PRE-EXISTING RESPIRATORY INFECTION ON THE COURSE OF ANOTHER SUPERIMPOSED BY THE RESPIRATORY ROUTE

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THERE is a large body of experimental evidence concerning alteration in the resistance of a host to infectious disease by modification of its diet, hormone balance, physical environment, etc. On the other hand, knowledge about the effect of pre-existing infection on the reaction of a host to a superimposed infection of another kind is very limited. Certainly there is little known about the mechanisms that may lead either to a synergic type of action of the two invaders or to increased resistance on the part of the host to one or the other.

From at least as early as the 16th century claims were made that "one ill cureth another". However, during the late 19th century and early in the present one a mass of literature accumulated on the subject of inhibitory action by one organism on another. An extensive review of this work is given by Florey and others (1949). While the greater part of this effort seems to have been devoted to antagonisms demonstrated by *in vitro* technique, there is also much writing on attempts to inhibit a pre-existing infection by exposing the host by various means to another pathogen. In this second field it seems that success or failure followed the experimenter, in good measure at least, according to his general beliefs supported by his "clinical impressions". Perhaps for this reason alone, efforts eventually ceased and there seems to have been no serious attention given to the problem from the experimental angle until the classical researches of Shope (1931 : 1936) on swine influenza. He clearly established that *Haemophilus influenzae* can precipitate acute infection with the virus of swine influenza but the bacterium produces no pathological change when acting alone. Subsequently a large body of evidence has accumulated concerning the activation of latent or superimposed bacterial infection by the influenza or pneumonia viruses. This can be exemplified by the work of Brightman (1935) and Glover (1941) with *Streptococcus pyogenes* in the ferret, and that of Volkert, Pierce, Horsfall and Dubos (1947) with *Mycobacterium tuberculosis* in the mouse. There has also been a reawakening of interest in the possible inhibition of one infection by the action *in vivo* of a second pathogen. But attention has been directed principally to such interference phenomena as occur in virus infections (reviewed by Lennette, 1951). A few sporadic findings in the field of bacterial disease clearly indicate that antagonisms can also occur but the researches have not been primarily designed to investigate the general problem. Thus, for example, Pullinger (1936; 1938) showed that when a combined injection of living tubercle bacilli and brucella organisms was made in the guinea-pig, the tubercle bacilli induced a local non-specific immunity response which impeded or completely prevented the

establishment of brucella infection. Again in a study of the efficacy of staphylococcal vaccines and their specificity, Cowan (1939) showed that a killed suspension of *Pasteurella pseudotuberculosis rodentium* gave the same order of protection against virulent staphylococci as any of the specific vaccines tested. Much more recently Mika, Goodlow, Victor and Braun (1954) and Nyka (1956) have shown that in the guinea-pig a primary infection with *Brucella* sp. confers a non-specific resistance in the host to a superimposed infection with *Coxiella burneti* (Mika *et al.*) or *Myco. tuberculosis* (Nyka).

Our interests have been centred mainly on problems connected with experimental respiratory infection. So far the guinea-pig has been the only host used. The anatomy of its respiratory tract is particularly well suited to studies on the influence of inhaled particle size on infectivity. Thus, for example, Harper and Morton (1953) have shown that the percentage retention of particles of different size found in the various areas of the respiratory tract of the guinea-pig follows closely that found by other workers on retention of particles in the human tract. This is why we selected the guinea-pig for earlier studies, accepting as a result serious limitation on the number and type of pathogens that could be tested, for the species seems susceptible to relatively few of the respiratory diseases of major interest. Nevertheless, it seemed that by such studies certain broad principles concerning modes of initiation of respiratory disease might emerge; and it now seemed of interest to examine the effect of those same pathogens acting together.

In the present work five species of pathogen have been studied. *Brucella suis* was used to initiate a relatively chronic form of disease and *Myco. tuberculosis* on a much smaller scale. *Pasteurella pestis*, *Bacillus anthracis* and *Str. pyogenes* (Group C) which normally can produce a fatal septicaemia by the respiratory tract, were used as the secondary invaders in animals suffering from either brucellosis or tuberculosis. In addition, some experiments were made in which *Br. suis* was superimposed on tuberculosis and *vice versa*. Finally, a few experiments were made using a relatively avirulent strain of *Str. pyogenes* (Group C) as the primary invader.

METHODS

Techniques for cloud production and assessment

These were as previously described (Henderson, 1952; Druett and May, 1952; Druett, Henderson, Packman and Peacock, 1953).

Organisms and their preparation for use

Earlier records give details concerning the strains of *Br. suis*, *P. pestis* and *B. anthracis* that were used, as well as methods for the production of suspensions (Druett, Henderson and Peacock, 1956*a*; Druett, Robinson, Henderson, Packman and Peacock, 1956*b*; Druett *et al.*, 1953).

One bovine strain (Vallée) of *Myco. tuberculosis* kindly supplied by Dr. Pierce of the Rockefeller Institute was used. Details for the preparation of suspensions for spraying, cloud collection and assessment have been given by Albericci and Fletcher (1956).

Two strains (K.159 and D.181) of *Str. pyogenes* (Group C) were kindly supplied by Dr. Lancefield of the Rockefeller Institute. Strain K.159 (relabelled S1) is relatively avirulent and was isolated from guinea-pigs during an outbreak of cervical lymphadenitis. Strain D.181 (relabelled S2) is the original strain 4 of Seastone (1939) and is virulent. They were laid down as freeze-dried cultures. Surface growth was obtained on 10 per cent horse blood agar (tryptic meat base). This medium maintained the strains in mucoid form. In preparing suspensions for spraying, a tryptic meat broth was used to which was added 1 per cent

Marmite, 0·6 per cent K_2HPO_4, 1 per cent xylose and 5 per cent horse serum, pH 7·4. Isolated mucoid colonies from an overnight growth at 37° were inoculated into 10 ml. of liquid medium and incubated for 7 hr. at 37°. A loopful of this culture was streaked on blood agar to check colonial form and the remainder used to inoculate 100 ml. of liquid medium which was then incubated at 37° for 18 hr. Pure mucoid forms were then obtained with a viable count of about 10^8 units per ml. For the production of clouds of the virulent strain, dilutions of this material were made in 10 per cent CCY broth (Gladstone and Fildes, 1940) in phosphate buffer, pH 7·2. Several drops of olive oil were added before spraying to avoid foaming. Cloud samples were collected in CCY diluent to which 0·05 per cent sodium alginate was added to avoid aggregation of particles during the collecting process (Henderson, 1952); a drop or two of olive oil was also added. On spraying, a sharp rise in viable count occurred owing to disaggregation of chains of cocci. After 4 min. the count became practically constant at a level of about eleven times that of the starting material and microscopic examination of the suspension showed organisms in groups of 2 to 5. Viable counts were made on tryptic meat agar containing 5 per cent horse serum. There was no loss in viability in suspensions as a result of prolonged refluxing during spraying and the undiluted culture could be stored satisfactorily at 0°–4° for at least a week.

Animals

Guinea-pigs weighing 350–400 g. were used. In all experiments they were allocated to their groups by random sampling. After exposure they were housed four to a cage, measuring approximately 40 × 40 × 24 cm. high. During much of the work they received a so-called complete diet in pellet form. This was done to avoid dust and insects, but it was later found that the diet was deficient and some deaths occurred some weeks after the animals had been exposed to a primary infection with *Br. suis*, an otherwise very rare event. Good hay of timothy grass was used as a supplement in later experiments and the trouble ceased. The results obtained in these latter experiments in no way invalidated the earlier findings.

All animals were autopsied at death or at appropriate times after infection. Various organs were cultivated and as necessary a selective medium was used for any particular organism involved.

Selective culture media

In experiments involving *Br. suis*, *P. pestis*, *B. anthracis* and *Str. pyogenes*, it was fortunate that highly selective media for the first three organisms were available. That for *B. anthracis* has already been described (Morris, 1955); those for *Br. suis* and *P. pestis* are to be described later by Mr. E. J. Morris of this Department. Where *Str. pyogenes* was involved, incorporation of 1/100,000 brilliant green to tryptose agar medium allowed ready growth of the streptococcus but completely inhibited *B. anthracis*. Where *Br. suis* and *P. pestis* were involved with the streptococcus they were grown on the respective selective media and the more rapid growth of *Str. pyogenes* on tryptic meat agar made the incorporation of inhibitors for *Br. suis* and *P. pestis* unnecessary. Experiments involving *Myco. tuberculosis* with other pathogens called for no selective media. Under the selected conditions of test macro- and microscopic evidence of infection with tubercle bacilli were adequate guides and because of the slowness of growth of *Myco. tuberculosis* no problem arose in detecting the presence of any accompanying pathogen.

RESULTS

Br. suis *as the Primary Invader*

Earlier work (Druett *et al.*, 1956*a*) showed that an estimated respiratory retention of about 60 single cells of *Br. suis* produced about 90 per cent infection. Harper (1955) has shown that 2–3 days after exposure to such small doses there is already an increase in the number of organisms recoverable from the lung. Infection of bronchial glands can be detected as early as the 4th day. From the 8th to 10th day practically all animals that will become infected yield brucella from bronchial glands and a few animals already give a positive culture from the spleen. At about 21 days there is a well marked generalised infection which reaches its

peak towards the end of the 4th week. Thereafter it declines so that by about the 18th week very few organisms are recoverable from the spleen.

More recently we have shown that the cellular response in the lung is primarily in lymphoid and lymphatic tissue and that the substance of the lung is not involved—at least not until very late in the disease. The first histological change is found about the 10th day after exposure. There is then an infiltration of mononuclear cells round the various lymphatics and foci of lymphoid tissue along the course of the main bronchi and blood vessels. Most of the cells are lymphocytes, macrophages and reticular cells. This process steadily increases until about 5 weeks after exposure, during which time there is a great increase in lymphoid tissue but there is no evidence of necrosis. The bronchial lymph nodes undergo a similar sequence of changes but these are followed by necrosis shown in the form of abscess formation with well marked peripheral fibrosis. By the 16th week after infection the whole lymphatic system is rapidly returning to normal. A few abscesses in lung tissue have by then developed and, as was shown by Harper (1955), this is accompanied by a sharp recurrent rise in the number of viable brucella organisms recoverable from the lung but the animals appear healthy and steadily gain weight.

Experiments with B. anthracis *as secondary invader*

In the first experiments guinea-pigs were exposed to a cloud of single *Br. suis* organisms estimated to give a 90 per cent infection rate. Eight days later they were exposed to a cloud of anthrax spores which for normal animals was estimated to give a 90 per cent death rate. The anthrax ran a normal course and there was no indication of reduction in the number of animals dying over those in control groups. In other experiments the initial dose of *Br. suis* was 100-fold higher but thereafter the animals were similarly treated; this procedure also failed to influence the course or degree of anthrax infection.

When experiments were made on exactly similar lines but exposure to anthrax spores was delayed for 21 days after exposure to *Br. suis* a marked reduction in death rate from anthrax was observed. The expected degree of about 90 per cent infection with brucella was found and there was a 95 per cent death rate in anthrax controls. In contrast, in the combined group only about 18 per cent of animals died with combined anthrax and brucella infection, one animal found negative for brucellosis died of anthrax, and one died having a positive brucellosis infection but culture of heart's blood and spleen was negative for anthrax. Survivors were autopsied 24 days after exposure to anthrax spores and none showed evidence of infection with *B. anthracis* on culture of spleen, liver, cervical or bronchial glands; all showed positive generalised brucellosis. Results of this type are readily reproducible. Table I summarises the combined data from three experiments of which one is that described above; the findings are self-evident.

Effect of route of infection

It was of interest to examine whether the route of primary or secondary infection altered the general course of events just described. In some experiments animals were first infected with *Br. suis* by the subcutaneous route and later exposed to a cloud of anthrax spores. In others, animals were exposed to a cloud of *Br. suis* and later challenged with anthrax spores by the intracutaneous route.

In one experiment guinea-pigs received an estimated dose of 22 cells of *Br. suis* subcutaneously in the flank. This was known to be about 4 ID_{50} and in the present experiment it was shown that of 10 controls receiving approximately 4 cells subcutaneously 6 showed generalised brucellosis 21 days later and 4 were negative. At this time an estimated LD_{90} of anthrax spores was superimposed by the respiratory route on the animals that had received 22 brucella organisms. The results are summarised in Table II; it happens that they form an almost exact replica of those given in Table I, thus indicating that primary infection by the respiratory route with brucella is not a necessary condition for the development of resistance to anthrax.

TABLE I.—*Combined Data from 3 Experiments showing the Inhibitory Action of Active Brucellosis on Superimposed Respiratory Infection with* B. anthracis

Animals exposed to :	Number of guinea-pigs. Tested.	Died.	Autopsy of survivors.
B*	30	0	27+B
B plus A	60	10+B+A	42+B
		5+A−B	2−B
		1+B−A	..
A	60	54+A	Not examined

Animals exposed to anthrax spores 21 days after respiratory infection with *Br. suis*.
*B = *Br. suis*. A = *B. anthracis*. + = positive culture. − = negative culture.
Experiments terminated 24 days after challenge with anthrax spores.

TABLE II.—*Influence of Route of Primary Infection with Brucella by Subcutaneous Route on Incidence of Anthrax Superimposed by the Respiratory Route*

Animals exposed to :	Number of guinea-pigs. Tested.	Died.	Autopsy of survivors 31 days after exposure to A.
B*	10	0	9+B
B plus A	20	3+B+A	14+B
		1+A−B	1−B
		1+B−A	..
A	20	17	Not examined

Infections : *Br. suis ca.* 4 ID_{50} subcutaneously.
B. anthracis spores *ca.* LD_{90} as a single spore cloud.
Anthrax superimposed 21 days after infection with *Br. suis*.
* B = *Br. suis*. A = *B. anthracis*. + = positive culture. − = negative culture.

The experiments designed to test the effects of respiratory brucellosis on the course of anthrax induced intracutaneously failed to show any significant degree of protection. The strain of *B. anthracis* used was highly virulent by this route; probably one or two spores were enough to kill. In one experiment the test group of animals first received an estimated ID_{90} dose of *Br. suis* and 21 days later each received a dose of 16 spores. All (20) control animals and 14/20 test animals died of anthrax within 23 days after challenge when the experiment was terminated. This result seemed suggestive but subsequent experiments showed a completely negative response although the challenge dose of spores was kept low (10 spores). These findings are discussed later.

Attempts at passive transfer of protection

Even the most sensitive type of serological test, as for example the agar diffusion precipitation technique of Ouchterlony, fails to show any antigenic relationship between *Br. suis* and *B. anthracis*. Nevertheless, it seemed important to test if the serum from animals with brucellosis could protect normal animals against respiratory infection with *B. anthracis*. It does not do so. In one experiment animals with brucellosis of 21 days standing were anaesthetised and bled from the heart. The blood from each was allowed to clot for 30 min. at 37°; the tubes were then "ringed" and placed at 0–4° overnight. The serum was decanted and spun to remove blood cells. Immediately thereafter 4 ml. of each sample (still heavily infected with *Br. suis*) was given subcutaneously to a corresponding normal guinea-pig. Twenty-four hours later these animals were exposed to an estimated LD_{90} of anthrax spores by the respiratory route. Concurrently a group of animals each receiving 4 ml. of normal guinea-pig serum in like manner and a group with acute brucellosis of 21 days' duration were similarly exposed to anthrax. The results are summarised in Table III. It is clear that no protection was given by serum from animals with brucellosis but the usual protective effect was evident in animals suffering from this disease. This type of experiment was repeated using a "pooled" and filtered brucella serum, each test animal receiving 8 ml. subcutaneously 24 hr. before challenge with anthrax spores. There was no evidence of passive protection, although again animals with active brucellosis exposed to the challenge dose showed the usual degree of resistance. This finding is discussed later.

TABLE III.—*Experiment on Non-specific Passive Protection against Respiratory Anthrax with Serum from Guinea-pigs with Brucellosis*

Preliminary treatment of animals.	Animals exposed to :	Number of animals : Tested.	Died.
4 ml. B* serum	A	20	19+A
4 ml. normal serum	A	20	16+A
Nil	B then A at 21 days	20	3+B+A
Nil	B	10	10+B
Nil	A	20	18+A

* B = *Br. suis*. A = *B. anthracis*. + = positive culture.

The state of immunity to anthrax in animals with brucellosis after surviving exposure to anthrax spores

It seemed of interest to test if a specific anthrax immunity response could be detected in these circumstances. Two groups of animals with brucellosis of 3 weeks' duration were, as in previous experiments, exposed to anthrax spores (*a*) by the respiratory route and (*b*) intracutaneously. Fig. 1 summarises the results which fully confirm the earlier findings; no effective protection against intracutaneous challenge was found, but a high degree of protection against challenge by the respiratory route was obtained. Five weeks later (*i.e.*, 8 weeks after the initiation of brucellosis) the survivors in the group exposed to respiratory challenge with anthrax spores were injected with spores by the intracutaneous route. The results, summarised in Fig. 2, at least suggest that some animals had acquired specific immunity to anthrax. Thus, while 17/20 control animals (with brucellosis

of 8 weeks' duration) died of anthrax, only 8/19 in the test group so died. Attempts were made to show the presence of circulating antitoxin in both test groups, but the method used (Belton and Henderson, 1956) failed to detect it; however, as noted in the paper quoted, the technique is known to have limited sensitivity.

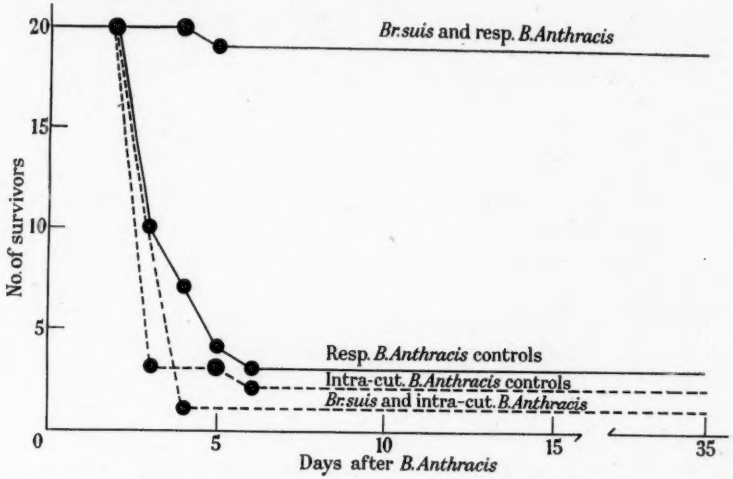

FIG. 1.—Effect of a primary respiratory infection of *Br. suis* on a secondary infection of *B. anthracis* given by the respiratory and intracutaneous routes.

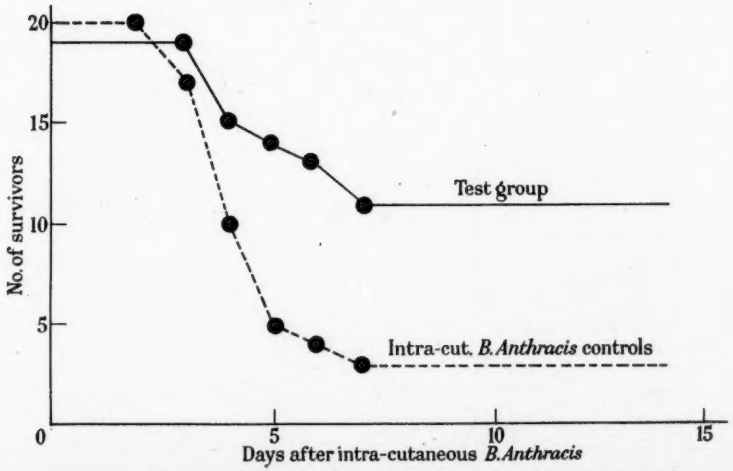

FIG. 2.—Experiment showing that animals with brucellosis which exhibit non-specific resistance to respiratory challenge with anthrax spores thereafter develop a specific immunity to anthrax.

Experiments with P. pestis *as secondary invader*

Earlier work (Druett *et al.*, 1956*b*) had shown that two forms of plague, both originating in the respiratory tract of the guinea-pig, can develop according to the size of the particle carrying *P. pestis* presented to the host. Small particles initiate a broncho-pneumonia which eventually leads to septicaemia and death. Large particles establish a septicaemia and death results more quickly without the development of pneumonia.

In the first experiment on combined infection, therefore, both small (single cell) and large (about 12μ diameter) particle clouds of *P. pestis* were used. As in the experiments with *B. anthracis*, it was found that 8 days after infection with single cell clouds of *Br. suis*, guinea-pigs had failed to develop any resistance to moderate doses (LD_{80-90}) of *P. pestis* delivered either as small or as large particles; this applied even if the initial dose of *Br. suis* was high (*ca.* 100 ID_{90}). However, 21 days after exposure to *Br. suis* protection against superimposed plague could be demonstrated.

With a high infective dose (100 ID_{90}) of *Br. suis*, animals exposed to a cloud of single cells of *P. pestis* showed delay in death time over that in controls but there was no significant degree of protection. If 12μ diameter particles of *P. pestis* were used to challenge similarly infected animals there was about a 75 per cent survival rate. The results of a typical experiment are shown in Table IV.

TABLE IV.—*Effect of Brucellosis Established by the Respiratory Route on the Development of Infection with* P. pestis *Similarly Applied*

Animals exposed to :	Number of guinea-pigs. Tested.	Died.	Autopsy of survivors (21 days after *P. pestis* challenge).
B*	17	0	15+B
B + P (single cell particles)	30	26+P+B	1+B−P
		3+P−B	
B + P (12 μ diam. particles)	30	5+P+B	11+B
		2+P−B	2−B−P
P (single cell particles)	25	25+P	..
P (12 μ diam. particles)	25	23+P	2−P

Initial infecting dose *Br. suis* by respiratory route = 100 ID_{90}.

Animals exposed to *P. pestis* 21 days after infection with *Br. suis* (single cell cloud).

* B = *Br. suis*. P = *P. pestis*. + = positive culture. − = negative culture.

In an isolated experiment it was found, as might now be expected, that if animals were exposed to a cloud of 12μ diameter *Br. suis* particles and later to a similar one of *P. pestis* then a high degree of protection resulted. Groups of 20 animals were used; 95 per cent of brucella controls were found infected and 85 per cent of plague controls died, whereas in the combined infection group 85 per cent of animals survived the plague challenge.

Inhibitory action of plague-infected tissue on the growth of Br. suis

At death or at autopsy of animals on the termination of an experiment various tissues were cultivated on selective media mentioned earlier to confirm the presence or absence of active infection with the selected pathogen. The procedure was to rub a cut tissue surface over selective agar. Animals with brucellosis of 3 weeks' duration yield a confluent growth on selective agar when tissues such

as lung and spleen are used. It was soon noticed, however, if such animals died of superimposed plague the growth of brucella was never confluent, frequently very scanty and confined to discrete colonies at the periphery of the smear, or entirely absent. These characteristics followed no regular pattern, except perhaps the order of inhibition in any animal was probably most strongly noticeable in lung, less so in bronchial and cervical lymph glands and least in spleen; but exceptions occurred. The phenomenon was further investigated. First it was shown that an *in vitro*-grown culture of the strain of *P. pestis* used had no inhibitory action on the growth of *Br. suis* when taken from *in vitro* culture. (The tests were performed by the simple procedure of streaking plates of selective agar with *Br. suis* and cross-streaking with *P. pestis*—which, of course, was itself inhibited in growth.) Likewise it was shown by the use of a medium highly selective for *P. pestis* that *Br. suis* had no inhibitory action on this organism. Then it was found that if the lung of an animal dead from uncomplicated plague was streaked at right angles across a similar smear of either *in vitro* or *in vivo*-grown *Br. suis* on its selective medium an area of complete inhibition of growth occurred at the crossing zone, but here the converse did not hold; *in vivo*-grown brucella failed to show any trace of inhibition for *P. pestis* (*in vitro*- or *in vivo*-grown) on selective medium for the latter organism.

It would seem from these observations that the factor(s) suppressing the development of plague in animals with brucella infection is distinct from the plague factor(s) which suppresses growth of *Br. suis*. However, this particular phase of the work requires special treatment which it has not yet been given. It is mentioned primarily to draw attention to the manifold factors probably at work in processes of combined infection.

Myco. tuberculosis *as secondary invader*

Two experiments were made in which *Myco. tuberculosis* was superimposed by the respiratory route on animals already suffering from brucellosis initiated by the same route. Unfortunately, at the time, the ID_{50} with single cell clouds of our strain of *Myco. tuberculosis* had not been defined. It is now known that the dose given contained about 500 ID_{99+} and was sufficiently severe to produce the first deaths among control guinea-pigs about 4 weeks after infection. All animals in the combined infection groups developed tuberculosis but in the early stages of the experiment there was clear macro- and microscopical evidence of a marked retardation in the development of tuberculosis over that in control groups. Thus it was not until about 5 weeks after infection with *Myco. tuberculosis* that the lesions in control and test animals showed about the same degree of severity.

More recently an experiment was made in which guinea-pigs with brucellosis were exposed to a cloud of single units of tubercle bacilli so that each animal had an estimated intake of 24 units. This probably represents about 20 I.D. As in the earlier experiments, tuberculosis was detected by culture in all animals by the 5th week after exposure but again there was a very definite delay in development over that in controls. Thus, for example, at the 4th and 5th week after exposure to tubercle 10/10 control animals on each occasion showed macroscopic evidence of tubercle formation in lung tissue, whereas only 2/10 were similarly affected in each of the brucellosis groups. By the 6th week 7/10 test animals showed evidence of tubercle formation whereas the controls were not only positive

but much more advanced. The experiment is still in progress 15 weeks after exposure but no deaths from tuberculosis have yet occurred.

Str. pyogenes *as secondary invader*

The virulent Group C strain is highly invasive by the respiratory route when presented to the guinea-pig as a cloud of small particles. In exposed animals abscesses began to form by the second day after exposure. These are preceded by cellular infiltration in the alveoli at the edge of the lung. Septicaemia later develops, very frequently accompanied by a pericarditis. Death supervenes between the 6th and 10th day after exposure.

Guinea-pigs with pre-existing brucellosis induced by the respiratory route proved to have no detectable alteration of response to a superimposed streptococcal infection of this group: a finding reminiscent of the results obtained with single cell clouds of *P. pestis*. Unfortunately we have been unable to extend the experiment as with *P. pestis* to the use of clouds of large particle size; highly irregular response to infection so far obtains with such clouds.

Myco. tuberculosis *as Primary Invader*

It is now known that an estimated intake of the order of 10 single cell units of our strain of bovine *Myco. tuberculosis* will produce about 100 per cent infection in guinea-pigs. This fact emerged after primary experiments on combined infection with *Myco. tuberculosis* and *Br. suis* or *B. anthracis* had been planned. It is now known that in these experiments a high dose (order of 500 ID) of tubercle bacilli was given. This massive primary infection may somehow have influenced results, but it remains that the protective effect produced by the primary tuberculous infection three weeks after exposure was closely similar in degree to that obtained with brucellosis against anthrax or plague. Table V summarises the results of a typical experiment with anthrax and Table VI of one in which *Br. suis* was the secondary invader. First it is seen that the dose of *B. anthracis* given killed 90 per cent of controls but only 15 per cent of those in the combined group died with an anthrax septicaemia. Again the dose of *Br. suis* was sufficient to infect 85 per cent of controls but in the combined group, tested at the second, third, fourth and fifth week after superimposing brucellosis, only 12·5 per cent of animals were found to suffer from this infection.

TABLE V.—*Effect of Established Pulmonary Tuberculosis on the Development of Anthrax by the Respiratory Route*

Animals exposed to:	Number of guinea-pigs. Tested.	Died.	Autopsy of survivors.
Tb*	20	2+Tb	18+Tb
Tb plus A	20	3+Tb+A	16+Tb
		1+Tb−A	..
A	20	18+A	..

Animals exposed to anthrax 21 days after infection with *Myco. tuberculosis*.
* Tb = *Myco. tuberculosis*. A = *B. anthracis*. + = positive culture. − = negative culture.
Experiment terminated 21 days after challenge with spores.

TABLE VI.—*Effect of Established Pulmonary Tuberculosis on the Development of Brucellosis by the Respiratory Route*

Animals exposed to :	Guinea-pigs examined : weeks after exposure to *Br. suis*. 2.	3.	4.	5.	Deaths from Tb.
Tb*	10/10+Tb	10/10+Tb	10/10+Tb	10/10+Tb	8/10 (8–14 weeks after exposure to Tb)
Tb plus B	10/10+Tb (1/10+B)	10/10+Tb (1/10+B)	10/10+Tb (1/10+B)	10/10+Tb (2/10+B)	*Nil*
B	*Nil*	*Nil*	*Nil*	17/20+B	*Nil*

Animals exposed to *Br. suis* 21 days after respiratory infection with *Myco. tuberculosis*.
* Tb = *Myco. tuberculosis*. B = *Br. suis*. + = positive culture.

Str. pyogenes (*Group C*) *as Primary Invader*

Experiments of this nature were made for two reasons. First the organism is a natural pathogen of the guinea-pig, producing a chronic lymphadenitis. Second, Seastone (1939) observed in an infected colony of guinea-pigs what appeared to be a spontaneous variation to high virulence of the otherwise lowly virulent invader, but most important he showed that active infection with a strain of low virulence induced a high degree of resistance to superimposed infection with a virulent one. However, this resistance could not be passively transferred by serum nor would various types of killed vaccine protect. The possibility remained, therefore, that while the two strains were probably genetically related the resistance conferred on guinea-pigs against infection with the virulent strain was of the same type as that reported in the present paper. In fact, Seastone states that the type of resistance he was dealing with might be " an uncomplicated form of so-called tissue immunity and should furnish a favourable approach to that important problem."

Attempts were made to infect guinea-pigs by the respiratory route with the strain of low virulence (S1); the results were erratic even when high doses were used and attention was turned to infection by the parenteral route (intracutaneous, as used by Seastone). Regular and non-lethal infection (within the period of the experiments) was obtained with an inoculum of about 1×10^8 chains of cocci in 0·2 ml. Twenty-one days later readily palpable induration of the superficial inguinal and axillary lymphatic glands had developed. At this stage animals were exposed by various routes to a secondary infection of either the virulent streptococcus Group C, *B. anthracis*, *Br. suis* or *P. pestis*.

Virulent streptococcus as secondary invader

In the first instance we attempted to repeat Seastone's (1939) work by re-infecting with the virulent strain (S2) of streptococcus; but, in addition to intracutaneous challenge, other groups of animals were tested by the intraperitoneal and inhalation routes. The results are summarised in Table VII. Confirmation is given to the finding that infection with S1 confers a high degree of protection against S2 given intracutaneously. A not significantly different degree of protection was observed when the animals were challenged by the intraperitoneal or inhalation route. The surviving animals were autopsied 27 days after secondary challenge. Cultures were made from inguinal and axillary lymph glands, heart's blood, spleen and lung tissue. All inguinal and axillary

glands were positive and yielded a growth of the S1 strain (which, with experience, could be differentiated from the S2 strain). Only 3 animals (from the group exposed to respiratory challenge with S2) yielded positive but scant growth of S2 from the heart's blood. Lung, cervical and bronchial lymph glands and spleen gave no growth.

TABLE VII.—*Effect of a Primary Low Grade Group C (S1) Streptococcal Infection in Guinea-pigs on Subsequent Exposure to a Virulent Strain of the Same Group (S2).*

Animals exposed to :	Number of guinea-pigs. Tested.	Died.	% deaths.
S1 with S2 given I.C.	30	2	7
S1 „ „ „ I.P.	40	1	2·5
S1 „ „ „ Inhal.	40	6	15
S2 given I.C.	20	18	90
S2 „ I.P.	20	17	85
S2 „ Inhal.	30	26	86

S1 given intracutaneously 21 days before applying S2. I.C. = intracutaneously. I.P = intraperitoneally. Inhal. = inhalation

B. anthracis, Br. suis *or* P. pestis *as secondary invaders*

The low grade streptococcal infection induced by the intracutaneous route, as described above, produced no signs of non-specific protection against any of these secondary invaders introduced by the respiratory route; this applied to *P. pestis* whether presented as a cloud of single cells or as a cloud of large particles. In addition, an experiment was made in which, 21 days after infection with the streptococcus, an estimated ID_{90} of *Br. suis* was injected intracutaneously at a site in close proximity to that used for injecting the streptococcus. At autopsy 4 weeks later there was no evidence of inhibition of infection with *Br. suis* but this finding may be akin to that observed with *B. anthracis* given intracutaneously to animals with brucellosis.

DISCUSSION

In earlier work, detailed study had been made of factors determining the initiation of infection by the respiratory route with *B. anthracis*, *Br. suis* and *P. pestis* (Druett *et al.*, 1953, 1956*a*, 1956*b*; Ross, 1957; Harper and Morton, 1953; Harper, 1955). The information thus obtained has been of great assistance in analysing the results of the present experiments on combined infection.

A point of first importance is that none of the experiments showed any evidence of enhanced pathogenicity as a result of superimposing one infection on another. Secondly, there was no evidence to support the records from early times that a pre-existing infection could be suppressed by another superimposed. It is, of course, possible that these negative findings are a matter of chance related to the host species and the combination of pathogens chosen for experimentation. Only two main positive findings emerged. First a pre-existing infection with one pathogen can induce a high degree of non-specific resistance to attack by a second and wholly unrelated one, while under other conditions using the same organisms no trace of resistance is observed.

Essential conditions for the development and manifestation of non-specific resistance seem to be, first, the ability of the primary invader to produce early non-fatal generalised infection of the lymphatic system, and second that the pathogen superimposed will be obliged to attempt entry and multiplication in

the host primarily through the lymphatic system. This is very clearly indicated in the experiments with *Br. suis* as primary invader and *B. anthracis* or *P. pestis* as secondary pathogens. The situation is in some measure reminiscent of the numerous observations on local tissue immunity. It differs, however, by the fact that a resistance can be demonstrated when the secondary invader is given by an entirely different route than that chosen for initiating primary infection; thus, for example, *Br. suis* given subcutaneously induces resistance to infection with *B. anthracis* or *P. pestis* secondarily given by the respiratory route.

From present experiments and earlier work noted above, there is good evidence to show that *Br. suis* given by the respiratory route invades primarily through the local lymphatic tissue. This is true also for *B. anthracis* (Barnes, 1947; Ross, 1957). The evidence is that, once brucellosis is thus established, a high proportion of animals are resistant to infection with *B. anthracis* imposed by the same route. On the other hand, if *B. anthracis* is superimposed by, say, the intracutaneous route no trace of non-specific resistance is detected. There is evidence to support the view that in this latter circumstance *B. anthracis* finds a nidus for continued multiplication external to the lymphatic system. This, of course, was Besredka's opinion; he mistakenly insisted that the only portal of entry for this organism was through the skin. Under these circumstances, therefore, it is probably not surprising that the non-specific resistance of the lymphatic system is eventually overcome.

The findings with *P. pestis* as secondary invader are not dissimilar. When this organism is presented to the host in the form of a cloud of single cells there is a primary infection of lung tissue leading consecutively to broncho-pneumonia, septicaemia and death. Under these circumstances there is no evidence (other than some delay in time of death) of non-specific resistance in animals with brucellosis. On the other hand, when *P. pestis* is superimposed as a cloud of large (*ca.* 12μ diameter) particles in animals with brucellosis a high degree of non-specific resistance to this secondary invader is observed. If the hypothesis outlined above is correct, these findings might be predicted from our knowledge of the pathogenesis of plague according to the route of infection. Thus we have shown (Druett *et al.*, 1956*b*) that if a cloud of single organisms of *P. pestis* is used, direct penetration of lung tissue occurs and generalised infection *viâ* the lymphatic system is by-passed. If a cloud of large particles is used, local penetration of the upper respiratory mucosa occurs but the main path for systemic invasion is through the local lymphatic system.

In experiments where *Br. suis* was the primary invader and *Myco. tuberculosis* or virulent *Str. pyogenes* Group C was superimposed by the respiratory route using clouds of small particle size, there was no evidence of effective non-specific resistance; both pathogens, of course, can invade lung tissue directly. However, there was marked delay in development of infection (over that in controls) when *Myco. tuberculosis* was superimposed on animals with brucellosis. So far we are at a loss to account for this but it is hoped that the detailed histological studies now in progress on the general development of tuberculosis in such animals may provide a clue.

Myco. tuberculosis as a primary invader led to a high degree of non-specific resistance against *B. anthracis*. It is also interesting that *Br. suis* used as secondary invader was almost completely inhibited. These findings are in general agreement with our hypothesis. Histological examination showed that under the conditions

we used, *Myco. tuberculosis* had stimulated a very marked hyperplasia of the lung lymphatic tissue by the time the potential secondary invaders were applied.

The results with *Str. pyogenes* Group C as primary invader are interesting in two respects. First, the lymphadenitis established completely failed to induce non-specific resistance to secondary infection with *P. pestis*, *B. anthracis* or *Br. suis* when these organisms were presented by the respiratory route under optimal conditions for the demonstration of resistance in the presence, say, of pre-existing brucellosis or tuberculosis. Second, we confirmed and extended Seastone's findings that such streptococcal lymphadenitis conferred a high degree of protection against superimposed infection with a fully virulent *Str. pyogenes* Group C. It seems clear, therefore, that this resistance is much more probably specific rather than non-specific as Seastone argued. The reasons for the failure to demonstrate non-specific resistance are not yet clearly understood. However, the pathological and bacteriological findings so far indicate that the low grade streptococcus infection may induce only strictly localised infection and lymphatic tissue response; this could be the determining factor. On the other hand, more recent experiments show no evidence of non-specific resistance to infection with *Br. suis* when the latter is superimposed by the intracutaneous route at a site in close proximity to one inoculated three weeks previously with the streptococcus of low virulence. Here again, however, parenteral injection of *Br. suis* may lead to conditions closely approximating those observed with *B. anthracis* in so far, at any rate, as failure to demonstrate non-specific resistance is concerned.

More attention could profitably be given to the finding that guinea-pigs with brucellosis which have survived a respiratory challenge with anthrax spores show evidence of the development of specific active immunity. The fact that this was demonstrated, as it were, by subterfuge is probably not for the present important, for the species is notoriously difficult to immunise against the disease under any circumstances. The results do not show complete protection and they are probably not statistically significant, but anyone with experience of this form of disease in the guinea-pig, and its prevention, might nevertheless regard the issue as highly significant.

The evidence which shows that tissue extract from animals dead from plague is strongly inhibitory to the growth of *Br. suis in vitro* is of interest in two respects. First it may offer a link with past history when claims were made that " one ill cureth another ". If this be so, it is here just unfortunate that the host died, as it were, at the hands of its potential saviour. Second, the phenomenon could not be shown to operate in reverse, *i.e.*, extract of tissues from animals with brucellosis failed to inhibit the growth of *P. pestis in vitro* and yet, of course, under certain circumstances the host with brucellosis was highly resistant to infection with *P. pestis*. As already noted, all these observations do is to indicate the manifold complexities in processes of combined infection. They also help to focus attention on the fact that the present experiments do nothing more than expose some of these complexities. So far they have not contributed to an understanding of the mechanisms involved.

SUMMARY

A primary infection with a pathogen such as *Br. suis* or *Myco. tuberculosis* which induces a marked generalised lymphatic response has been shown to produce non-specific protection against secondary infection with organisms

whose normal attack is through the lymphatic system. No protection is observed against secondary invaders of this nature if presented in such a way that they attack the host otherwise than primarily through lymphatic tissue. The mechanisms involved in the resistance process have not been elucidated but some of the factors determining whether the process will develop or manifest itself have been disclosed.

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THE ENZYMIC ACTIVITY OF INFLUENZA VIRUSES

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A LARGE number of viruses give the phenomenon of haemagglutination but they vary widely in respect of the species of red blood cells which they can agglutinate and the conditions under which cell agglutination will occur. Those of the influenza group, which includes the viruses of mumps and of Newcastle disease of fowls, appear to be unique in possessing an enzyme capable of destroying the receptors of susceptible red cells so that, under appropriate conditions, the stage of virus adsorption is followed by spontaneous virus elution (Hirst, 1941, 1942). The adsorption-elution cycle has been shown to occur also with host-tissue cells susceptible to infection (Hirst, 1943 ; Fazekas de St. Groth, 1948 ; Stone, 1948). It is therefore reasonable to suppose that the virus enzyme performs some useful function in the infection process, although there is no definite proof that this is so, and the rôle of the enzyme remains to be elucidated.

There have been a few reports of differences between influenza virus strains in their elution behaviour but no serious attempts to correlate enzymic activity with other biological attributes like infectivity and toxicity. Briody (1948) found that, with the exception of one strain (WSE), all those tested eluted completely from human red cells within 10 minutes at 37°. On the other hand Sigel (1949), using fowl cells, demonstrated different elution rates at either room temperature or 4°, even with virus strains isolated from the same outbreak of influenza. Such apparent contradictions are not surprising in view of the diversity of techniques used by different workers. In a previous publication (Sheffield, Smith and Belyavin, 1954) it was shown that the speed and degree of virus elution are largely dependent on the amount of virus adsorbed (the cell loading) and the concentration of red cells in the elution mixture. Other factors are the variability of red cells from different donors, their deterioration during storage, the reaction temperature and the nature of the suspending medium. Comparative studies of the enzymic activities of different virus strains, therefore, require rigorously standardised and controlled experimental conditions. The present paper reports the results of such studies and represents the first stage of an investigation designed to eludicate the biological significance of the virus enzyme.

MATERIALS AND METHODS

Viruses

One type A and four A prime strains were used.

PR8 is one of the classical type A viruses, isolated by Francis in 1934. There is no doubt that many substrains with somewhat different characteristics are now extant. The strain used is one which has been maintained in our laboratory since 1947 by regular serial egg passage by the allantoic route of inoculation.

HAL is an A prime strain isolated in 1951 by amniotic inoculation of chick embryos. Two distinct lines were established by adaptation to the allantoic cavity after 4 and after 40 serial amniotic passes to give respectively substrains HAL/AL and HAL/AM.

Ash and Last are two A prime viruses isolated in 1953 and adapted to the allantoic cavity after 2 and 4 amniotic passes respectively.

421/53 is an allantoically adapted 1953 A prime strain obtained from the Virus Reference Laboratory, P.H.L.S., Colindale.

Seed stocks of each virus were laid down at −70°. Infected allantoic fluids were obtained as required by inoculation of 11-day embryonated eggs with 0·2 ml. seed virus diluted 10^{-3} or 10^{-4} in nutrient broth containing penicillin (2500 u./ml.) and streptomycin (3000 μg./ml). Infected eggs were incubated at 35° for 48 hr. and thoroughly chilled at 4° before harvesting. The fluids were kept up to a maximum period of 7 days at 4°.

Red cell suspensions

Human red blood cells were obtained from Group O donors. After separation from the plasma they were washed thrice in physiological saline and stored at 4° up to 7 days. For experiments 0·5 per cent suspensions in saline were made, final adjustment of concentration being effected by means of a photo-electric densitometer.

Fowl cell suspensions were prepared in the same manner from blood withdrawn by venepuncture into citrate saline, a rota of fowl donors being maintained for the purpose. Fowl cells were always used within two days of their collection.

Enumerations of eleven separate standardised 0·5 per cent suspensions of each type of cell gave average counts of 89×10^7 per ml. for human cells and 72×10^7 per ml. for fowl cells.

Haemagglutination (HA) titrations

These were done by a modification of the Hirst-Pickels photo-electric densitometer method (Hirst and Pickels, 1942; Belyavin, Westwood, Please and Smith, 1951).

Virus adsorption and elution

In all comparisons of two or more viruses, or different preparations of the same virus, the test fluids were first adjusted by dilution to give approximately equal HA titres. Mixtures of equal volumes, usually 20 ml., of chilled virus and chilled red cell suspension were made and held at 4° for 30 min. to allow maximum virus adsorption. They were then centrifuged at 2000 r.p.m./3 min. and the cells re-suspended in 10 ml. saline previously warmed to 37°. The suspensions were held in a water bath at 37°, samples withdrawn at appropriate intervals being immediately centrifuged at 2000 r.p.m./3 min. The supernates representing the eluate samples, the supernatant fluid from the adsorption stage and a sample of original untreated virus were stored overnight at 4° and titrated in parallel on the following day. Elution yields were calculated as percentages of the amounts of virus which had been adsorbed by the red cells.

Infectivity titrations

Eleven-day embryonated eggs were inoculated allantoically with 0·2 ml. quantities of 10-fold dilutions of the test fluids in penicillin-streptomycin broth. For each dilution 5 or 7 eggs were used. They were incubated for 72 hr. at 35°. A sample of undiluted allantoic fluid from each egg was tested by the haemagglutination pattern test in W.H.O. plastic trays using fowl red cells; definite aggregation of the cells was accepted as evidence of egg infection.

Toxicity titrations

Test fractions were first concentrated by centrifugation for 20 min. in a Spinco centrifuge at 40,000 **g**, deposits being re-suspended in 50 per cent broth saline to give a concentration factor of about × 70. The concentrates were then adjusted by appropriate dilution so that HA titres were as nearly equal as possible and further three-fold dilutions made. Groups of mice, usually 10 per dilution, were inoculated intracerebrally with 0·03 ml. volumes. Those showing any sign of abnormality during the first 18 hr. were discarded; thereafter they were examined twice daily up to the 96th hour after inoculation. They were twirled by the tail

once daily to induce the onset of convulsions in animals with cerebral lesions. Deaths within this period were regarded as being due to toxicity of the inoculum. Toxicity indices were calculated by the method of Thompson's moving averages (Thompson, 1947).

EXPERIMENTAL

Efficiency of Virus Enzyme in Relation to Species of Red Cells

In 39 experiments the rates of elution of one or other of the virus strains from both human and fowl cells were tested in parallel. The elution curves for each strain varied from experiment to experiment. This is to be expected with different batches of a virus and with cells obtained from different donors. In

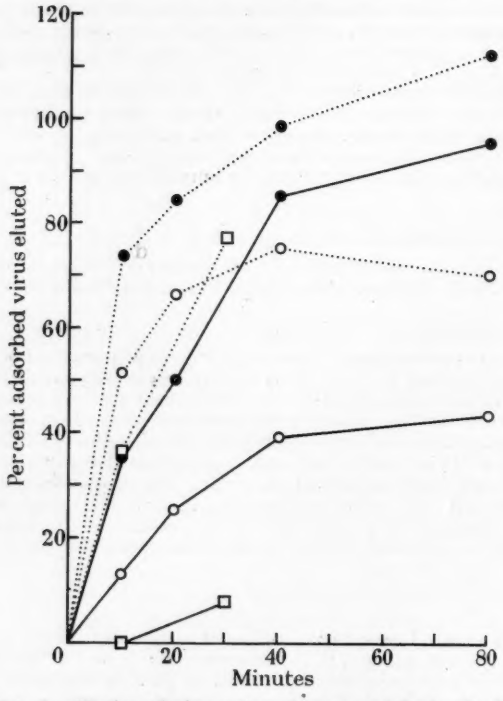

FIG. 1.—Elution of viruses from human and fowl red cells.
——— Human cells. - - - - - - Fowl cells. ● PR8. ○ Ash. □ HAL.

every experiment without exception, however, the rate and degree of elution from fowl cells was appreciably greater than from human cells. Differences were uniformly greater with the A prime viruses than with PR8 except for strain 421/53 which behaved like PR8. The other A prime strains, Ash, HAL and Last, always eluted slowly and incompletely from human cells, often showing almost no elution at all. Representative experiments with PR8 and two A prime strains are illustrated in Fig. 1.

This striking difference of virus enzymic activity on human and fowl cells was not due to more efficient adsorption of virus by the latter. In all the experiments adsorption was shown to be practically complete so that, with standardised cell suspensions, the ratio of virus particles to number of cells (the cell loading) must have been approximately equal for each type of cell. The difference must reflect a greater susceptibility of fowl cell substrate to the virus enzymes. The result was totally unexpected in view of the fact that many freshly isolated virus strains agglutinate human cells to high titre but only acquire the capacity to agglutinate fowl cells after allantoic passage in eggs. Clearly, enzymic efficiency is independent of the receptor affinities between virus and cell.

Two phenomena illustrated in Fig. 1 occurred occasionally, namely the apparent recovery of more virus than had been adsorbed and a lower recovery at 80 min. than at 40 min. The most probable explanations are, respectively, some disaggregation of virus clumps and the readsorption of a little eluted virus by intact cell receptors during late stages of an experiment. Neither of these phenomena invalidates the general conclusions.

Elution Efficiency as a Characteristic of Virus Strains

The elution curves obtained from the numerous comparisons between human and fowl red cells suggested that one of the intrinsic characteristics of a virus strain might be its elution efficiency. Direct comparisons of strains were made by obtaining their elution curves in parallel under identical conditions. Although it had become clear that any differences would be much more clearly revealed with human cells than with fowl cells both types were included in each experiment. The complexity of the experiments was such as to preclude comparisons between more than three strains at one time. In each experiment approximately equal cell loadings were ensured by adjusting the virus preparations to equivalent HA titres before their adsorption.

The results of six experiments are summarised in Table I. In four the classical type A PR8 virus was compared with the A prime strain Ash. Without exception human cells revealed a striking difference between their elution efficiencies both as regards speed and completeness of elution. In the first three experiments samples were withdrawn at intervals up to 60 or 80 min. but, as it became apparent from these and many other tests not recorded in the Table that elution is practically always virtually complete in 40 min. so that samples withdrawn at 10 min. and 40 min. are sufficient to characterise the elution behaviour of strains, later experiments were thus curtailed. In experiments 5 and 6 A prime strains, Ash, HAL and Last, were directly compared. All gave poor elution from human cells, sometimes failing to elute at all. In contrast the other A prime strain 421/53, included in experiment 3, behaved like PR8 virus.

The results with fowl cells fully confirmed the data of Fig. 1 ; elution of all the viruses was far better than from human cells and failed to give consistent differences which would serve to differentiate them.

The Effect of Egg Passage on Virus Elution Behaviour

The PR8 strain used had been maintained for several years by serial allantoic passage. The passage history of the only rapidly eluting A prime strain 421/53, was unknown. The other three A prime strains, with relatively poor elution

efficiency, Ash, HAL and Last, had undergone only two or three allantoic passes since their isolation from cases of influenza by amniotic egg inoculation. This suggested that high activity of virus enzyme on human red cell substrate might depend on the selection of a mutant during serial allantoic passage. Strains Ash, HAL and Last were therefore subjected to such passage and seed virus obtained at different stages was used to prepare batches of infected fluids for comparison. Strain Ash was investigated by a series of nine comparative experiments which are summarised in Table II. These were not merely repetitions with the same batches of virus but tests of different batches obtained at different times by inoculating eggs with seed virus of the preceding allantoic passage.

In seven experiments 5th pass virus eluted better than 2nd pass virus and in two experiments no significant difference was apparent. Continued passage failed to increase further the elution efficiency of Ash, for virus batches of the 12th, 17th and 18th passes were no better than those of the 5th pass ; indeed in experiments 7 and 8, they were appreciably worse. Strains HAL and Last failed to show any effect of serial allantoic passage. With HAL 2nd passage batches were compared with both 12th and 30th passage batches ; with Last, 3rd and 12th passes were compared. None gave any elution whatever at 10 min. whilst at 40 min. percentage elution varied from 0 to 25 in different tests but no significant differences between early and late passes were shown. The attempt to convert one of the A prime strains into a stable rapidly eluting virus was therefore completely unsuccessful.

The effect of long continued amniotic passage was also investigated with strain HAL. Seeds representing the 4th and 40th serial amniotic passes subsequent to amniotic isolation of the virus were obtained. From these, batches of infected allantoic fluid were prepared by two allantoic passes which were necessary to provide sufficient material for elution experiments. Two comparative tests were done in each of which both human and fowl red cells were used. The results are shown in Table III. Neither early nor late passage virus gave any elution whatever from human cells but the result of each experiment indicated that prolonged amniotic passage had reduced enzymic activity against fowl cells.

The Fractionation of Infected Fluids into Components of Different Enzymic Activities

The most striking feature of the experiments with Ash virus recorded in Table II, is the variable behaviour of both 2nd and 5th passage virus from experiment to experiment. For example, the percentage elution of 2nd pass virus at 40 min. ranged from 0 to 42, and of 3rd pass virus from 16 to 89. Different susceptibilities of red cells from different donors are not responsible because each passage virus varied independently although a single red cell suspension was always used for their comparison. Even PR8 virus exhibits similar variability although to a lesser degree. The most probable explanation, which was accepted as a working hypothesis, is that virus in an infective allantoic fluid is not homogeneous in enzymic potency, so that the activity of any batch is determined by the relative proportion of different types of virus particles. It is well known that such fluids are heterogeneous in other respects, containing so called " complete " highly infective virus and " incomplete " virus with a relatively low infectivity/

TABLE I.—*The Elution Behaviour of Different Virus Strains from Human and Fowl Red Cells*

Expt.	Virus strain.	Elution from human cells (minutes). 10.	20.	40.	60.	80.	Elution from fowl cells (minutes). 10.	20.	40.	60.	80.
1	PR8	35	50	85	—	95	73	84	98	—	>100
	Ash	0	22	19	—	38	>100	>100	89	—	100
2	PR8	43	54	54	—	59	61	73	89	—	57
	Ash	0	0	3	—	0	20	23	30	—	29
3	PR8	23	43	50	60	—	48	62	64	59	—
	Ash	6	10	20	30	—	62	69	86	71	—
	421/53	27	62	81	100	—	76	90	96	96	—
4	PR8	26	—	52	—	—	40	—	82	—	—
	Ash	0	—	0	—	—	11	—	39	—	—
5	Ash	18	—	31	—	—	103	—	86	—	—
	HAL	0	—	4	—	—	65	—	67	—	—
	Last	4	—	22	—	—	80	—	96	—	—
6	HAL	0	—	0	—	—	25	—	47	—	—
	Last	0	—	10	—	—	46	—	50	—	—

Elution figures in all Tables are the percentages of adsorbed virus recovered by elution at times stated.

TABLE II.—*Effect of Allantoic Passage of Strain Ash on Elution Efficiency*

	Number of consecutive allantoic passes: 2		5		12		17		18	
Elution time (minutes):	10.	40.	10.	40.	10.	40.	10.	40.	10.	40.
Expt. No.										
1	0	18	13	39	—	—	—	—	—	—
2	16	15	30	55	22	56	—	—	—	—
3	8	35	22	41	19	40	—	—	—	—
4	0	13	19	89	—	—	—	—	—	—
5	18	31	41	55	—	—	—	—	—	—
6	0	0	15	40	—	—	—	—	—	—
7	8	42	25	62	—	—	—	—	13	48
8	15	42	14	32	—	—	0	19	—	—
9	0	13	0	16	—	—	0	22	—	—

TABLE III.—*Effect of Amniotic Passage of Strain HAL on Elution Efficiency*

Expt.	No. of amniotic passes.	Elution from: Human cells at minutes. 10.	40.	Fowl cells at minutes. 10.	40.
1	4	0	0	27	58
	40	0	0	0	20
2	4	0	0	31	49
	40	0	0	0	15

haemagglutination ratio. " Incomplete " virus, however, has never been separated from the " complete " form.

The problem was attacked by attempting to fractionate infected fluids into rapid and slow eluting components. For the purpose Ash 5 virus was chosen because of its variable elution behaviour coupled with sufficient activity to give a reasonable chance of obtaining adequate supplies of the different components.

The fractionation cycle

Infected allantoic fluid was mixed with an equal volume of 0·5 per cent or 0·25 per cent suspension of human red cells in saline. The strength of cell suspension was varied to ensure adequate cell loading with virus batches of different HA titres. The mixture was held at 4° for 30 min. and then centrifuged. The cells carrying adsorbed virus were re-suspended in a volume of saline at 37° equal to either a half or a quarter of the original volume of allantoic fluid. After 10 min. the cells were deposited by centrifugation and the supernatant fluid containing the rapidly eluting virus (10-min. fraction) removed. The cells were again re-suspended in the same volume of saline to allow elution to proceed for a further 30 min. and then again deposited. The supernatant fluid from this stage, containing virus eluted between 10 and 40 min. was discarded. The residual adsorbed virus was stripped from the cells by suspending them in saline containing RDE (receptor-destroying enzyme from *vibrio cholerae*) and holding the suspension at 37° for 30 min. The supernatant fluid from a final centrifugation constituted the " RDE fraction ".

Comparative tests of fractions

The elution behaviours of the 10-minute and RDE fractions were examined in parallel by the standard type of comparative elution experiment. In some experiments a sample of the original allantoic fluid was also included. Before adjustment of the test fluids to equivalent HA titres RDE was added to the allantoic fluid and 10-minute fraction, and sodium citrate to a final concentration of 4 per cent then added to each test fluid to inactivate the RDE. This was done in order to ensure that the elution of each fluid occurred as nearly as possible under identical conditions and to prevent interference by RDE with the normal virus adsorption and elution mechanism.

Eight experiments were done with human red cells as the test substrate. Elution was allowed to proceed for an hour, samples being withdrawn at 10, 20, 40 and 60 min. Fig. 2 shows the elution curves obtained by plotting percentage virus recoveries against elution times. Without exception the 10-minute fraction gave much more rapid and more complete virus elution than the RDE fraction. In view of the variability from experiment to experiment it is particularly striking that the curves of the least active of all the 10-minute fractions and the most active of all the RDE fractions are widely separated.

To avoid confusion, curves for the original allantoic fluids which were included in five experiments are omitted from the Figure. Three of them fell between the curves of their 10-minute and RDE fractions. This also occurred in several subsequent experiments designed for other purposes. Anomalous results however were obtained on two occasions, once when allantoic fluid gave even better elution than its 10-minute fraction and once when it gave worse elution than the RDE fraction. The reason for this quite exceptional behaviour is unknown.

Two similar experiments were done with fowl cells. In both, the elution of the 10-minute fraction was appreciably more rapid and more complete than that of the RDE fraction although the difference was not nearly as great with human cells. Allantoic fluid was included in only one of these tests ; its elution curve fell between those of its fractions.

These experiments show conclusively that infective allantoic fluids contain virus particles with different degrees of enzymic activity. It is clear from the elution curves that in this series of fractionations both the 10-minute and RDE fractions were still heterogeneous but contained very different proportions of rapidly eluting and slowly eluting particles. The production of fractions of equal haemagglutination titres but with widely different enzymic activities by this technique afforded a means of investigating the relation between enzymic potency and other biological characteristics of the virus.

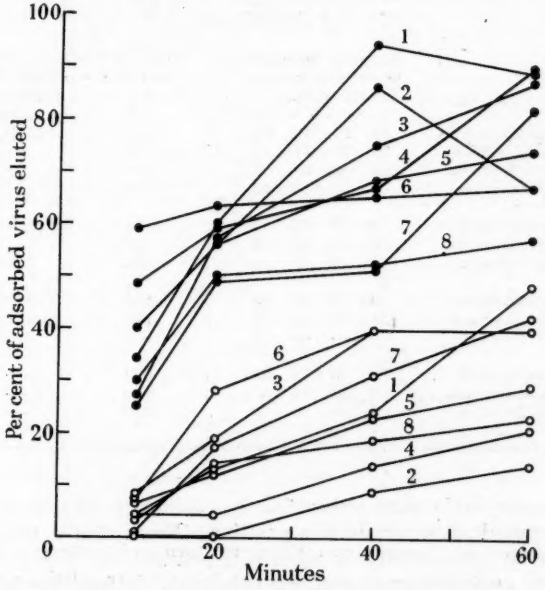

FIG. 2.—Enzymic activity of fractions from 8 infected allantoic fluids.
●———● Fractions obtained by elution for 10 min.
○———○ Fractions released from red cells by RDE.

The Relation between Enzymic Potency, Infectivity and Toxicity

Ten-minute and RDE fractions were obtained by the method described. Samples of original allantoic fluid and 10-minute fractions were held at 37° during the subsequent processing of the RDE fractions in order to equalise possible reductions of infectivity at this temperature. In one experiment each fraction was subjected to a second fractionation cycle with a view to obtaining more homogeneous preparations. Unfortunately the heavy losses of virus, which are inevitable in each cycle, resulted in final products with such low HA titres that they had to be concentrated by high speed centrifugation in order to test their elution behaviour and sufficient concentration for toxicity tests was not possible.

Toxicity effects in mice are only produced by relatively enormous doses of virus, so for these tests concentration of the fractions was always necessary. This was done as described above. The concentrates were stored at −70° for not longer than 14 days. For test they were rapidly thawed and equated to equivalent HA titres. They were used also for the infectivity titrations in eggs done on the same day.

The results of four experiments are presented in Table IV. It had been expected that the enzymically inactive virus of RDE fractions might represent partially degraded or " incomplete " virus which would have low infectivity and low toxicity. But, on the contrary, it was consistently at least as infective and significantly more toxic than the virus of the enzymically active 10-minute fraction. This was so even in the two experiments in which it gave no elution at all over the 60-minute period. Unprocessed allantoic fluid virus was both more

TABLE IV.—*The Correlation between Enzymic Efficiency, Infectivity and Toxicity of Ash Virus*

Expt.	Fractions.	Enzymic efficiency percentage elution in min. 10.	20.	40.	60.	Tests on fractions after concentration HA titre.	Infectivity titre Log. ID_{50}/ml.	Toxicity titre.
1	Allantoic fluid	36	36	41	48	1550	—	3·4
	10 min. fraction	59	63	65	67	1805	—	3·4
	RDE fraction	7	28	40	40	2290	—	4·2
2	Allantoic fluid	0	10	23	33	626	9·91	4·6
	10 min. fraction	8	24	42	53	732	8·91	1·4
	RDE fraction	0	0	0	8	696	9·46	2·6
3*	Allantoic fluid	19	25	39	60	134	9·10	—
	10 min. fraction	21	48	71	75	119	8·10	—
	RDE fraction	—	—	—	0	135	8·50	—
4	Allantoic fluid	0	0	12	22	678	—	6·5
	10 min. fraction	0	12	26	57	400	—	1·4
	RDE fraction	0	0	0	0	390	—	2·0

* Concentrates of fractions from 2 successive fractionation cycles used.

infective and more toxic than either fraction although its elution curve in each experiment occupied an intermediate position. From this it would appear that neither infectivity nor toxicity is wholly dependent on either HA activity or enzymic potency against human red cell substrate. Infectivity is the most labile of all virus activities and some degradation of an essential factor concerned in it may occur during fractionation. The elimination of all virus eluting between 10 and 40 min. must also be borne in mind. The significant difference between the toxicities of rapidly eluting and completely non-eluting fractions, however, strongly suggests that toxicity may be a function of those virus particles which are unable to release themselves from the tissue cells to which they have become adsorbed.

Enzymic Activity of Ash Virus Fractions against Egg White Inhibitor

In view of the greater susceptibility of fowl cell substrate to the virus enzyme when compared with human cells, comparative tests of infected allantoic fluid and its 10-minute and RDE elution fractions against egg white were done by a modification of the method of Lanni, Lanni and Beard (1951). A standard dose of each virus preparation was mixed with egg white diluted 1/10 in phosphate buffer pH 7·0 and held at 37°. Samples were removed at intervals and immediately boiled for 2 min. to inactivate the virus. All samples were titrated in parallel

by haemagglutination inhibition tests, using heated Lee virus as indicator, to estimate the residual egg white inhibitor.

A linear relationship between inhibitor destruction and square root of reaction time (Belyavin and Cohen, unpublished) allows direct comparison of the reaction velocities of virus preparations when tested under identical conditions. Several

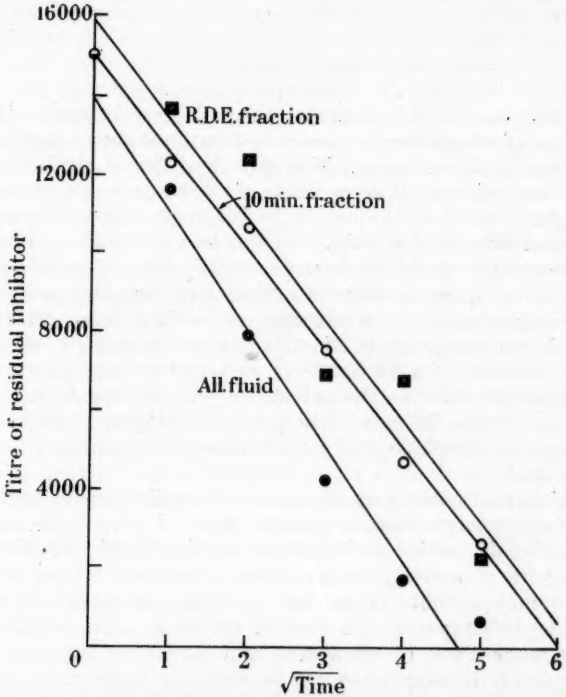

FIG. 3.—Activity of fraction concentrates on egg-white inhibitor. (The elution rates of these fractions are given in Table IV, Expt. 4).

● = Allantoic fluid = 5·6 AD/ml.
○ = 10 min. fraction = 3·4 AD/ml.
■ = RDE fraction = 3·4 AD/ml.

experiments, one of which is illustrated in Fig. 3, showed that egg-white inhibitor is destroyed at the same rate by allantoic fluid, 10-minute fraction and RDE fraction virus.

Egg Passage of 10-minute and RDE Fractions at Limit Dilution

If rapidly eluting and non-eluting virus particles represent stable entities with elution behaviour dependent on a genetic difference it should be possible to separate pure clones of each type by serial passage of 10-minute and RDE fractions at limit dilutions. Three serial passages of each fraction were done by

inoculating groups of 10 or 20 chick embryos with 10-fold dilutions. Eggs were incubated for 72 hr. At each pass, all allantoic fluids were harvested separately and tested for HA activity. Those found to have been infected at limit dilution were tested for elution rate from human red cells. Without exception the elution curves represented a mixture of rapid, slow and non-eluting virus particles. It is therefore improbable that elution efficiency is a stable genetic character.

DISCUSSION

The investigation has failed to establish the biological function of the receptor-destroying enzyme of the influenza viruses but has brought to light a number of phenomena of considerable interest. Not only do different strains differ in their enzymic activity but infected allantoic fluids of any single strain are heterogeneous in this respect. Such fluids are known to be heterogeneous in respect of particle infectivity; indeed this could not fail to be so in view of the cyclical nature of virus replication and the probable degradation of particles which are shed into the allantoic cavity at an early stage. Enzymic heterogeneity however is clearly not the result of degradation in the ordinary sense of the term. There is evidence that infectivity is the most labile of all virus characteristics, with toxicity a close second, but the virus of RDE fractions, although unable to elute from human red cells, is fully as infective as that of the rapidly eluting 10-minute fractions and is definitely more toxic for mice. A somewhat analogous situation is presented by the occurrence of morphologically dissimilar forms, spheres and filaments, in some infected fluids.

Two kinds of elution heterogeneity are theoretically possible either of which would account for the experimental results. The infective fluids might contain two types of virus only, active and inactive, or they might consist of particles with various degrees of activity ranging from extremely active to completely inactive. The second possibility is the more probable and would appear to derive support from the heterogeneity of elution fractions. On neither hypothesis however could a single cycle of adsorption and elution be expected to separate a fluid into completely homogeneous components, for some of the inactive virus must be released from red cells by the action to adjacent active particles (Hirst, 1949) while the RDE fraction is likely to be contaminated with active particles which have been occluded or readsorbed. Even by successive cycles of adsorption and elution it would not be possible to obtain a preparation consisting of highly active particles with equal elution efficiency, but one might expect in this way to obtain a fraction consisting entirely of particles inactive against the particular substrate used for the fractionations. This in fact seems to have been achieved in some experiments by only two fractionation cycles.

The differences in enzymic behaviour are not explicable on a simple quantitative basis. It is readily conceivable that splitting of an enzyme substrate, as presented by human red cells, might require a concentration of enzyme on the virus particles far greater than that required to split enzyme substrate in fowl cells or egg white, even if the substrates are absolutely identical, which is probably not so. RDE fractions, however, should then exhibit at least some reduction of efficiency against egg white inhibitor. The fact that they do not suggests that the virus enzyme may not be a single entity but a mixture of two or more, possibly

representing minor modifications of a basic pattern. An alternative explanation is that only one enzyme is concerned but that its spatial distribution in the virus particles differs so that its effectiveness is governed by the relative accessibility of the substrate in different carriers. This is in line with the influence of receptor configuration on haemagglutination behaviour demonstrated by Smith and Westwood (1950). Changes of spatial distribution during maturation of virus particles are readily conceivable and could result in a wide variety of surface patterns. This hypothesis would also account for the failure to produce enzymically homogeneous virus by either prolonged allantoic passage or passage at limit dilutions coupled with the demonstrable effect of passage on the relative proportions of rapidly eluting and non-eluting virus. It is significant that Bjorkman and Horsfall (1948) were able to alter the elution rate of Lee virus from fowl cells by treatment of the virus with lanthanum acetate or ultra-violet irradiation without loss of infectivity, although in their experiments the changed virus remained stable on passage.

The possibility that failure to elute might be responsible for toxic effects, due to interference with normal cell metabolism by virus permanently adsorbed on the cell surface, was first suggested by the observation that some virus strains with low enzymic potency were exceptionally toxic (unpublished observation). The greater toxicity of the RDE fractions lends some support to this view. Enzymic activity on human red cells, however, is not necessarily an indication of activity on the substrate presented by the tissue cells subject to toxic effects. The relative susceptibility of such tissue cells to 10-minute and RDE fractions might be quite different from the susceptibilities of human cells, fowl cells or egg white whether several enzymes or the spatial distribution of a single enzyme is involved. To establish a causal relationship between enzymic activity and toxicity studies with the tissues susceptible to virus toxicity such as mouse brain, on the lines of the experiments recorded, are indicated. Unfortunately, the technical difficulties involved preclude an investigation of this sort at present.

SUMMARY

Influenza virus strains differ in enzymic activity as measured by their elution rates from human and fowl red cells. The differences are revealed much more clearly by human cells than by fowl cells.

The virus particles in infective allantoic fluids are heterogeneous in respect of their elution efficiency. Such fluids can be fractionated to yield rapidly eluting and slowly eluting components. By means of RDE it is possible to obtain fractions consisting of virus which is quite unable to elute spontaneously from human red cells.

Non-eluting virus may be fully infective and even more toxic than rapidly eluting virus. It is suggested that toxicity for mice by the intracerebral route may possibly be a function of an inability of the virus to free itself from tissue cells to which it has become adsorbed.

Egg passage of virus fractions at limit dilutions gave no evidence that elution efficiency is a stable genetic character.

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SURVEY OF PAPERS

BARBARA M. EVANS and MACPHERSON administered to young rats a high-phosphate, high-calcium diet. They confirmed that on addition of the carbonanhydrase inhibitor acetazolamide (Diamox) nephrocalcinosis was produced. This could be correlated with a fall of the percentage of " chelated calcium " in the urine. There was no correlation between the degree of calcium deposition in the kidney and the level of total calcium or of citrate in the urine (p. 533).

CINADER and PEARCE examined the antigenic properties of two different ribonuclease preparations. Diffusion or electrophoresis in agar gels and sensitisation of tanned red cells preceded the application of antibodies. Although electrophoresis demonstrated two components in ribonuclease, this heterogeneity was not reflected in an antigenic heterogeneity. Molecular ratios for rabbit antibody and ribonuclease were 2·8 at antibody excess and 1·55 at equivalence (p. 541).

JANE SHELDON-PETERS and JANET VAUGHAN analysed calcium, nitrogen, hydroxyproline and hexosamine in the bones of rabbits six months or more after a single injection of ^{90}Sr. No great alteration was noted in the chemical composition of the bones of new-born or adult animals, but significantly larger amounts of hexosamine and smaller amounts of hydroxyproline were found in rabbits five to seven weeks old at the time of injection. This is considered to be due to the high uptake of ^{90}Sr in the epiphyseal regions of weanling rabbits, much of which is not resorbed and remains to cause radiation dysplasia. In younger animals, the uptake is more diffuse and much of it is reabsorbed by remodelling; in older rabbits there is less initial uptake owing to far less active growth (p. 553).

KAY finds that the rapid inactivation of a typhoid phage by phosphate, citrate or versene is largely prevented by bivalent metals, particularly calcium. Inactivation was found to be associated with the release of ^{32}P from labelled phage indicating a loss of nucleic acid; this was accompanied by collapse of the " head " of the particle seen in electron-micrographs. Phage can be inactivated by isolated receptor substance in the absence of calcium and may itself be a complex of calcium or magnesium ions (p. 560).

BILLINGHAM and BRENT attempted to transfer transplantation immunity against skin homografts with serum. Survival times of the grafts were not curtailed when compared with those on untreated animals. Variations in serum dosage, times and routes of administration did not alter the uniformly negative results. These findings are considered to support the view that transplantation immunity is transferable by cells and not by serum (p. 566).

JACKSON and BURROWS have found that many strains of *P. pestis*, when grown on a defined medium containing haemin, form dark brown colonies. After prolonged incubation pigmented colonies produce non-pigmented secondary colonies, which on subculture give only non-pigmented colonies. They describe a medium for differentiating pigmented and non-pigmented strains (p. 570).

JACKSON and BURROWS, in a continuation of the work presented in the preceding paper, have found that non-pigmented mutants of virulent strains have reduced virulence for mice. It is suggested that the reduction in virulence is associated with an inability to metabolise an iron compound, present in the mouse, which is utilised by virulent strains (p. 577).

GILLMAN and CHRISTINE GILBERT describe four types of arteritis and one of phlebitis which may develop rapidly after five days' administration of large doses of vitamin D to

rats. Treatment with thyroxin aggravated these lesions : previous thyroidectomy or treatment with cortisone diminished their severity. These changes are likened to others produced experimentally in animals and to some occurring in disease in man. It is suggested that the mechanism underlying all these conditions may be a derangement of renal function involving the metabolism of calcium and phosphorus (p. 584).

HENDERSON, LANCASTER, PACKMAN and PEACOCK have found that when infection is produced by inhalation in guinea-pigs the results of inoculation by the same route three weeks later with another organism never show enhancement of the secondary infection and often afford evidence of protection against it. Thus primary *Br. suis* infection protects against *B. anthracis*, *Myco. tuberculosis* and under certain conditions *P. pestis* : primary infection with *Myco. tuberculosis* protects against *Br. suis* and *B. anthracis*. There was no protection against Group C streptococcus infection, nor did this protect against others. Protection is believed to depend on involvement of the lymphatic system by the primary infection, and to be effective against another species invading by this route (p. 597).

WILSON SMITH and COHEN have investigated the elution rates from human and fowl red cells of influenza virus type A and of various A prime strains. The activity was always greater for fowl cells and the difference more marked with most of the A prime viruses. From allantoic fluids, slowly and rapidly eluting fractions could be separated. Virus which lacked the property of elution from human red cells could be released by RDE and was found to be fully infective, and abnormally toxic for the mouse brain (p. 612).

SURVEY OF PAPERS.

MIMS describes a series of studies on Rift Valley Fever virus infection of mice. This system is very suitable for precise quantitative studies and, it is suggested, may provide a convenient laboratory model for yellow fever in man or monkey.

1. Intracerebral or intravenous inoculation produced rapidly fatal infection in mice with very high titres of R.V.F. virus in the blood. By either route the virus was viscerotropic and intracerebral titrations were shown to give reproducible results (p. 99).

2. Infectivity titrations on blood and suspensions of carcass at intervals after inoculation with different amounts of virus showed that adsorption occurs mainly in the first hour and that liberation is detected after 5–9 hr. After a period varying with the dose, peak blood titres of much the same magnitude are reached. (p. 110).

3. The adsorption of virus from the blood after inoculation of different amounts was investigated. Virus taken up at this stage is not detectable and the log. of the amount unadsorbed is proportional to the log. dose. Even after very large doses, mice do not die until one cycle of growth is completed (p. 120).

4. Serial passage in mice of large inocula of R.V.F. virus yields low infectivity titres attributable to interference by incomplete virus particles which are antigenically active. High infective titres regularly reappear later during serial transfer and could be explained by the production of incomplete virus which is non-interfering (p. 129).

SMITH and GALLOP describe the isolation of a purified fraction from the thoracic and peritoneal exudates of guinea-pigs dying of anthrax, which has immunising and antiphagocytic activity. Observations are made on other substances present in the exudates (p. 144).

BELTON and HENDERSON have devised a method of titrating anthrax antibody which consists of determining the volume of a serum which will prevent the reaction in rabbit skin produced by a test dose of anthrax toxin derived from the plasma of guinea-pigs in the terminal stage of anthrax. Since the immunising antigen which can be extracted from appropriate cultures also combines with antibody, the potency of such an antigen can be determined by the same test, toxin being added to mixtures of antigen and antibody as an indicator of whether any of the antibody remains uncombined (p. 156).

TOBIN inoculated cotton rats with living or formalinised poliomyelitis virus suspensions of Types 1, 2 and 3, and later titrated antibody in their sera. All antibody produced was type-specific. When different strains isolated in Minnesota during 1946–54 were used, cross-neutralisation tests revealed no antigenic differences within each type. Some tests were done of the antigenic potency of vaccines in clinical use, a purpose for which the cotton rat appears suitable : a few similar tests were done in human subjects (p. 161).

BALDUCCI and TYRRELL describe the multiplication of *Toxoplasma gondii* in normal rabbit and monkey cells and in human malignant cells. Such cultures have been used as a source of complement-fixing antigen which has been used in diagnostic tests (p. 168).

STONER has used the method of internal calorimetry to measure the changes in the rat liver temperature and blood flow during the development of toxic hepatic necroses. The changes induced by dimethylnitrosamine, carbon tetrachloride, sodium fluoroacetate, beryllium sulphate and allyl formate support the view that the necroses do not result from alterations in the blood supply. He concludes that the toxins act by directly interfering with cellular metabolism (p. 176).

WEINBREN and BARBARA H. BILLING injected bilirubin intravenously in rats and at intervals determined the amount excreted in bile obtained by cannulating the common bile duct. The rate of excretion was determined both in normal animals and after partial hepatectomy: after this operation the rate of liver regeneration exceeded the rate of excretion for the first day, but thereafter increase in liver size and in excretory capacity proceeded in parallel (p. 199).

BALDUCCI, ELISABETH ZAIMAN and TYRRELL have searched the north of England for evidence of infection with influenza C and viruses of the APC group. Six APC viruses have been recovered from tissue cultures of adenoids, and complement-fixing and neutralising antibodies have been found in sera from adults suffering from acute febrile infections of the respiratory tract (p. 205).

SHILO, WOLMAN and WOLMAN have shown that intravenous, intraperitoneal or intracardiac injection of levan into rabbits and guinea-pigs inhibits the inflammatory response to staphylococci. The resulting lesion in levan-treated animals is a spreading haemorrhagic necrosis at the site of injection of the organisms (p. 219).

SURVEY OF PAPERS

CYMERMAN-CRAIG, RUBBO, LODER and BARBARA J. PIERSON have examined some thiosemicarbazones containing the diphenyl structure and some related thioureas for antituberculous activity. They found that although a number were active *in vitro*, they were ineffective *in vivo* (p. 1).

SHEFFIELD and WILSON SMITH have compared the dose-response curves for poliomyelitis (Lansing) virus in HeLa cell cultures with those in mouse brain. The cell cultures gave a more uniform response and this was shown not to be due to adaptation of the virus to that system. Replicate titrations, using five cultures for each dilution and an observation period of 8 days, gave reproducible results, but unexplained variations in titre were found with the same virus pool tested on different occasions (p. 5).

MOLLIE BARR has studied further the effect of different amounts of T.A.B. vaccine on the immune response of guinea-pigs to tetanus toxoid. Different doses of toxoid and of toxoid-T.A.B. mixtures, in which the concentration of vaccine varied, were given in two injections. With one batch of toxoid, antitoxin production was enhanced at high dosage levels with preparations incorporating the smaller amounts of vaccine. In similar experiments with a toxoid of poor immunogenic quality the vaccine either reduced the antitoxin response or had no effect (p. 11).

HOBSON inoculated mice intraperitoneally with *Salm. typhi murium* and treated them with oral furazolidone in different doses, either forthwith or after a delay, judging the effects by mortality and by estimates of the bacterial content of the organs. Only large doses given early eradicated the infection ; other systems of treatment overcame the acute stage, but the organism persisted in the spleen. Withdrawal of treatment in such mice was not followed by relapse (p. 20).

HEWITT has investigated an outbreak of renal tubular necrosis affecting mature male CBA mice and sparing females and weanlings of the same strain and albinos of both sexes. The cause was believed to be accidental exposure to chloroform vapour, and similar lesions were produced in male mice by experimental exposure to low concentrations of chloroform. It is pointed out that mice surviving unrecognised exposure to this hazard would be unsuitable for any studies in which renal function could play a part (p. 32).

FOURMAN, MCCANCE and PARKER made rats K-deficient by diet and exchange resin for 6–8 weeks, after which period the animals were allowed to recover on stock diet. They found progressive renal damage with tubular dilatation, glomerular atrophy and high blood urea up to 7 months after temporary K deficiency (p. 40).

SNELL and MCINTYRE have found a reduction in the histochemically demonstrable cholinesterase at the motor end plate from 11 to 45 days after section of sciatic nerves in guinea-pigs. This finding is considered in relation to the increased sensitivity to acetylcholine following nerve section (p. 44).

WOOD and WHITE claim to have produced acute or subacute diffuse glomerulo-nephritis in about half the mice they injected subcutaneously with heat-killed *P. mirabilis*. Using fluorescein-labelled antibody, antigenic material from *P. mirabilis* was found to be localised in the glomerular cells (p. 49).

BRIGITTE ASKONAS and WHITE injected guinea-pigs in the footpad with ovalbumin and examined various tissues after three weeks. By producing fluorescent conjugates of ovalbumin with its antibody in frozen tissue sections they were able to count the antibody-containing plasma cells. Tissue preparations were also incubated with ovalbumin and radioactive glycine *in vitro*. The degree of radioactivity found in the anti-ovalbumin after incubation was taken as an indication of the ability of that tissue to form new antibody. There was good correlation between the number of antibody-containing plasma cells in the different tissues and the ability to form antibody (the bone marrow being an exception). The highest activity was seen in lymph nodes remote from the site of injection, the spleen and the bone marrow (p. 61).

BELYAVIN has studied the distribution and properties of a substance in serum which inhibits the agglutination of influenza virus by antibody. It was shown to be present in human, rabbit, guinea-pig, horse, mouse and fowl serum. It acts indifferently against several types of influenza virus, is thermolabile and destroyed by trypsin but not by RDE. It is distinct from the haemagglutination inhibitor of normal serum (p. 75).

MAYERSBACH and PEARSE injected egg-white intraperitoneally in young female mice, and studied its distribution in the kidney, where it traversed the glomeruli and was re-absorbed in the proximal convoluted tubules. Of the three methods used for tracing it, two, fluorescent marking of the protein and the use of fluorescent antibody, gave concordant results; those obtained by staining and by histochemical methods were divergent (p. 81).

DICK, JANET S. F. NIVEN and GLEDHILL describe the properties and behaviour of a virus, closely related to mouse hepatitis virus, which they have isolated from mice inoculated with serum from a human case of infective hepatitis and with hypertonic glucose solution. This virus is referred to as MHV3 and is unrelated to human hepatitis (p. 90).

SURVEY OF PAPERS

ROWLEY has studied the early changes in susceptibility of animals to *Bact. coli* which result from the injection of cell walls from different Gram-negative bacilli. A rapid increase in susceptibility is replaced after 24 hours by a resistant state. These changes are attributed to the lipopolysaccharide fraction of the cell walls and are correlated with alterations in the bactericidal properties of the serum (p. 223).

HESTRIN and DAVIES have inhibited skin preparation in the local Shwartzman phenomenon by high polymer saccharides (levan and dextran). These substances also cause transitory leucopenia and depress diapedesis, and in the authors' view their findings agree with the hypothesis that leucocytic infiltration is necessary for skin preparation (p. 235).

MITCHISON used the induction of transplantation immunity to detect the iso-antigens of spleen cells transplanted into irradiated mice. The iso-antigens were detectable in some hosts after 51 days and it is suggested that colonisation of the organs of the host by the transplanted cells had taken place. The production of antibody by the transplanted cells was enhanced in irradiated hosts (p. 239).

SMITH produced retinal toxoplasmosis in rabbits by injection into the vitreous of a strain of toxoplasma isolated from a fatal human case in which there was an acute choroidoretinitis. The organism was demonstrable in the rabbit lesions and could be recovered from them (p. 248).

ELIZABETH PHEAR and RUEBNER have studied ammonium and amine production *in vitro* by different intestinal bacteria growing in media containing a mixture of amino acids. The addition of glucose usually increased the amount of growth and reduced ammonium and amine formation. When chlortetracycline was added in amounts which partially inhibited growth most, but not all, of the strains showed a proportional reduction in activity (p. 253).

SMITH, TEMPEST, STANLEY, PATRICIA HARRIS-SMITH and GALLOP claim that *B. anthracis* toxin found in the plasma of guinea-pigs dying of anthrax has at least two components. They have failed to prepare anthrax toxin *in vitro* using conditions approaching those occurring *in vivo* (p. 263).

CAVANAGH, HOWARD and WHITBY report a study of the action of the "neurotoxin" of *Sh. shigae* in different animals. All the effects seen appeared to have a primarily vascular basis. Lesions were produced in the central nervous system in mice and rabbits, in the alimentary tract in rats and in rabbits given large doses: in hamsters there were pleural effusions and pulmonary congestion. Guinea-pigs were unaffected (p. 272).

MACPHERSON describes the microscopical changes in the hearts of rats on a diet deficient in potassium and containing sodium sulphonic resin. The first changes occur at the end of the first week and while myocardial necrosis follows, connective tissue and blood vessels are not involved. The differences between this hypokalaemic necrosis and ischaemic lesions are emphasised (p. 279).

BURROWS and BACON have studied the effect of cultural conditions on the development of resistance to phagocytosis by *P. pestis in vitro*. The change appeared unrelated to capsule formation. Virulent organisms were shown under some conditions to produce a substance inhibiting the activity of mouse polynuclears against otherwise susceptible bacterial cells (p. 286).

HEGYESSY, BOZSÓKY and SCHULEK gave a combined typhoid-tetanus prophylactic to two groups of 12-year-old children, of which one group had previously been immunised on three occasions with typhoid vaccine alone. There was no difference in the tetanus antitoxin response of the two groups (p. 300).

DONIACH and LOGOTHETOPOULOS removed one half of the thyroid in rats and re-implanted it subcutaneously. One group was then given methyl-thiouracil in the drinking water, the other serving as a control. The auto-implants were transferred to another site after three, and again after four months. Sixteen months after the first operation all rats were killed. Implants survived in a majority of both groups. In animals treated with methylthiouracil they were histologically more active, but they showed no increased tendency to tumour formation (p. 306).

SURVEY OF PAPERS

SHOOTER and WYATT have extended their previous studies of the mineral requirements of *Staph. pyogenes* by observations on the effect of potassium. More thorough extraction of broth by passage through a larger amount of ion exchange resin produced a medium which permitted little growth even when adequate amounts of magnesium and calcium were added, and the effects of adding potassium to this showed that the organism also requires this element. The addition of potassium alone did not support growth. Observations were also made on the amount of potassium uptake and on changes in pH in the presence of different amounts of magnesium and calcium (p. 311).

DINGLE and PAGE THOMAS have compared the *in vitro* metabolism of normal synovial membrane with that from cases of rheumatoid arthritis: three types of the latter tissue are distinguished. Oxygen uptake, glucose utilisation and lactate production showed large increases over the normal, these being proportional to the activity of the disease (p. 318).

DOLBY and MARJORIE G. MACFARLANE have assessed the lethal potency for mice and the haemolytic and lecithinase activities of concentrated α-toxins from five strains of *Cl. welchii*. The ratios of lethal to enzymic activity varied with different toxins. Parallel differences in potency were found on comparing rates of haemolysis in varying concentrations of guinea-pig red cells with the lecithinase activity (p. 324).

BOYSE, MORGAN, PEARSON and PAYLING WRIGHT inoculated rabbits with a neurotropic strain of herpes virus and studied the distribution of the lesions produced by injecting rabbit plasma protein labelled with ^{131}I intravenously 20 hours before the animal was killed, and estimating radio-activity of different levels of the cord after death. This method depends on the escape of the protein from the blood vessels in inflamed areas. Inoculation with virus was by injection into the median, first lumbar spinal or sciatic nerve. The consequent distribution of radio-activity in the cord was consistent with its dispersion through tissue fluids from its level of entry rather than along axons (p. 333).

WIDDICOMBE, HUGHES and MAY inoculated rabbits with *B. anthracis*, cannulated lymph vessels draining the site of inoculation and made quantitative cultures at intervals from the lymph so obtained. The findings suggested that extensive multiplication of the bacilli occurs in lymph nodes, and that the eventual profuse discharge of these bacilli is the cause of the terminal bacteriaemia (p. 343).

LANGLEY, LODGE and WOODCOCK performed bilateral adrenalectomy in female rats and studied the effect on survival time of the strain of rat, of the temperature at which they were housed, and of simultaneous öophorectomy. They are chiefly concerned with the interpretation of data from such experiments when the median and mean survival times differ, and suggest methods applicable to this situation (p. 350).

HULTQUIST injected cobaltous chloride into rats. This caused an increase of the reduced glutathione in liver and pancreas, and a decrease in the kidney. Similar though quantitatively different changes followed the injection of synthaline A (p. 357).

SMITH, ZWARTOUW and PATRICIA W. HARRIS-SMITH fractionated *B. anthracis* grown *in vivo*, and determined the anti-phagocytic, virulence-enhancing, anti-complementary and anti-anthracidal activities of the products. Apart from capsular polyglutamic acid, they identified a protein which has anti-phagocytic activity, and is believed possibly to function as an "aggressin" (p. 361).

D'ARCY HART and REES previously reported that treatment of an avirulent tubercle bacillus with non-ionic surface-active agents induced cord formation but not virulence. They have now found that treatment of another avirulent strain with either Triton A20, as used before, or with a similar polyoxyethylene ether synthesised in their Institute, causes not only cord formation but a change in colony form, reduced dehydrogenase activity, and the acquisition of virulence. A suggested explanation of the inverse relationship between dehydrogenase activity and virulence (a closer relationship than that between cord formation and virulence) is that both may depend on cell permeability (p. 372).

NAGINGTON describes a quantitative study of the bactericidal action of immune serum on typhoid bacilli in the presence of complement, carried out in the cups of perspex haem-agglutination trays with a small inoculum, the survival of the bacilli being indicated by the reduction of triphenyltetrazolium chloride during subsequent incubation. A method for titrating the antibody is proposed which determines the highest dilution of the serum capable of killing the whole of an inoculum of about 10 bacilli in the presence of excess complement (p. 385).

NAGINGTON used the technique described in the preceding paper to examine the activity of bactericidal antibody in relation to the antigenic structure of the typhoid bacillus. The rôle of Vi, O and R antigens is discussed (p. 397).

FLECK has made experiments to confirm or refute the conclusion of Alice C. Evans that cross-protection can occur by anti-sera to different types of *Str. pyogenes*. Mouse protection tests with anti-sera of types 1, 6, 18 and 23 showed all to be type-specific except one of two type 6 sera, which protected against type 18 (these two types being within one of Evans' "immunogenic groups"); absorption with type 36 removed this protective power, which suggests that its action was due to an antibody other than anti-M. Similar results were obtained in tests of the phagocytosis-promoting power of these sera. It was also observed that human leucocytes destroy ingested streptococci whereas mouse leucocytes do not (p. 406).

MAXTED has studied the indirect bactericidal test as a means of identifying antibody to the M antigen of *Str. pyogenes*. He finds that this test makes it possible to examine strains which are avirulent for mice, and sera which give M-like precipitations with streptococcal extracts, probably due to non-specific antibodies (p. 415).

SURVEY OF PAPERS

MIMS and MASON, in further studies of Rift Valley Fever virus, find that a haemagglutinin can be obtained by acetone-ether extraction of infective serum. An inhibitor also present in the serum is unstable in the cold, and stored but otherwise untreated serum will therefore also give haemagglutination. Haemagglutinins obtained in these two ways differ in the species range of cells agglutinated, pH limits of activity and infectivity. Haemagglutination by this virus is not inhibited by yellow fever or Semliki Forest antisera (p. 423).

GOSTLING and BEDSON infected chick embryo mince or dispersed chick embryo cells with herpes simplex virus, and titrated the virus at intervals in the whole culture or in the washed and homogenised tissue from it. In some experiments extracellular virus was neutralised by adding antiserum to the medium. No evidence was obtained in favour of the hypothesis of an " eclipse " phase in virus multiplication (p. 434).

BAUER and BRADLEY have estimated the xanthine-oxidase activity in mouse brain infected with a wide range of different neurotropic viruses (47 strains). Increases of up to 13-fold over the activity of normal mouse brain were found, and the degree of increase was constant for each virus. It was possible on a quantitative basis to place the viruses in five groups comprising mainly agents having known relationships and it is suggested that this measurement may be useful taxonomically (p. 447).

BOYSE, KLEMPERER, MORGAN and PAYLING WRIGHT have recorded the pressure changes at different levels in the vertebral canal and in the posterior cranial fossa of rabbits. They found that the " arterial " pulsations are not synchronous throughout the cerebro-spinal axis, those in the thoracic region occurring earlier than those in the posterior cranial fossa and the lumbar region. They suggest that these asynchronous pressure fluctuations may be a factor in the dispersal of particles through the extracellular compartment of the central nervous system (p. 461).

REYNELL and SPRAY produced hepatic necrosis with CCl_4 and then measured absorption and transit of phenol red, glucose and ^{131}I through the alimentary tract of rats. The results suggest that in liver failure the motor functions (as measured by transit of these materials from one to another segment of the gut) are depressed earlier than failure of absorptive capacity (p. 466).

CAMERON has shown that the W-Swiss strain of mice can be sensitised to foreign proteins by doses as small as those effective in most other animals. He compared the action of serum and individual plasma proteins from different animals and studied the effect of different routes of injection and numbers of injections for sensitisation : the shocking dose had to be given intravenously. Shock could usually be prevented by giving an anti-histamine (p. 470).

LOTZ and HARRIS have studied chemotaxis in polymorphonuclear leucocytes by the photographic trace technique. Contrary to the findings of other workers, they have observed that chemotaxis can occur in the absence of complement, serum, glucose, calcium or magnesium, and that it is not inhibited by citrate or by concentrations of azide or fluoride insufficient to abolish motility altogether. Aluminium silicate was not negatively chemotactic. Wide variations in the tonicity of the medium were tolerated (p. 477).

BURROWS and BACON have shown that virulent strains of *P. pestis* and avirulent strains capable of developing resistance to phagocytosis can produce two antigens which other strains cannot. Sera containing antibody to these antigens render virulent organisms phagocytable, and interaction between these antigens and antibody can be demonstrated by the agar diffusion precipitation method (p. 481).

ROSEMARY D. SIMON has studied the fermentation reactions and pigment production in different media of 99 strains of *Ps. pyocyanea* and 30 of other *Pseudomonas* species. A peptone-free medium was used for fermentation tests, since otherwise alkali production masks the reaction. *Ps. pyocyanea* is distinguishable from other *Pseudomonas* species by two invariable characters which none of the latter exhibit, formation of pyocyanin and fermentation of D-arabinose. The strains examined could be assigned to 7 of Gessard's 16 types by his methods of studying pigment formation (p. 494).

ALBERT, REES and TOMLINSON have studied the antibacterial and other properties of a series of pyridine-N-oxides. The most active was 2-mercaptopyridine-N-oxide, the mode of action of which was investigated, and compared with that of oxine, which it was shown to resemble closely (p. 500).

HARRIS has applied to rat connective tissue cells a method which he recently used for measuring the respiration of macrophages. By this means it was shown that multiplication can continue normally in the absence of oxygen : metabolic inhibitors which interfere only with oxidative mechanisms did not affect it. Some possible fallacies in experiments supposed to have shown that anaerobiosis is fatal to these cells are pointed out (p. 512).

PHILLIPS and ELBERG have found that when either cobalt nitrate or ethylenediamine tetra-acetic acid is added to a culture medium, *Str. pyogenes* grown in it appears to have an enhanced virulence for mice. A similar increased mortality and shortening of the time to death were observed when these substances were added to an inoculum which had been grown in normal medium. Observations were made which suggest that their effect is not on the organism but on host resistance (p. 518).

MOLE compared the levels of liver glycogen in irradiated and non-irradiated rats, some of which had been adrenalectomised, castrated or adrenalectomised and treated with cortisone acetate. The rise in liver glycogen following irradiation was prevented by adrenalectomy shortly after irradiation, but not if low doses of cortisone were given to the adrenalectomised rats. He concludes that the evidence that irradiation stimulates adrenal activity has been misinterpreted and suggests that the irradiated rat uses cortisone more efficiently than controls (p. 528).

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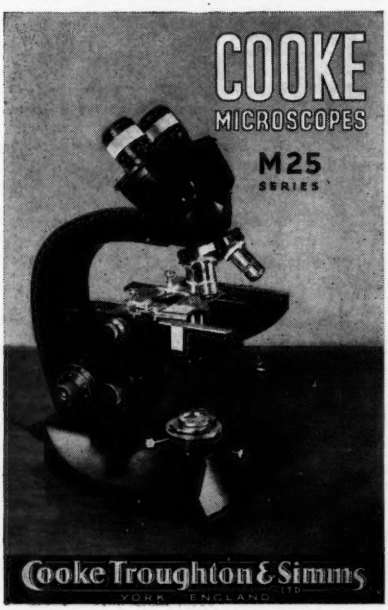

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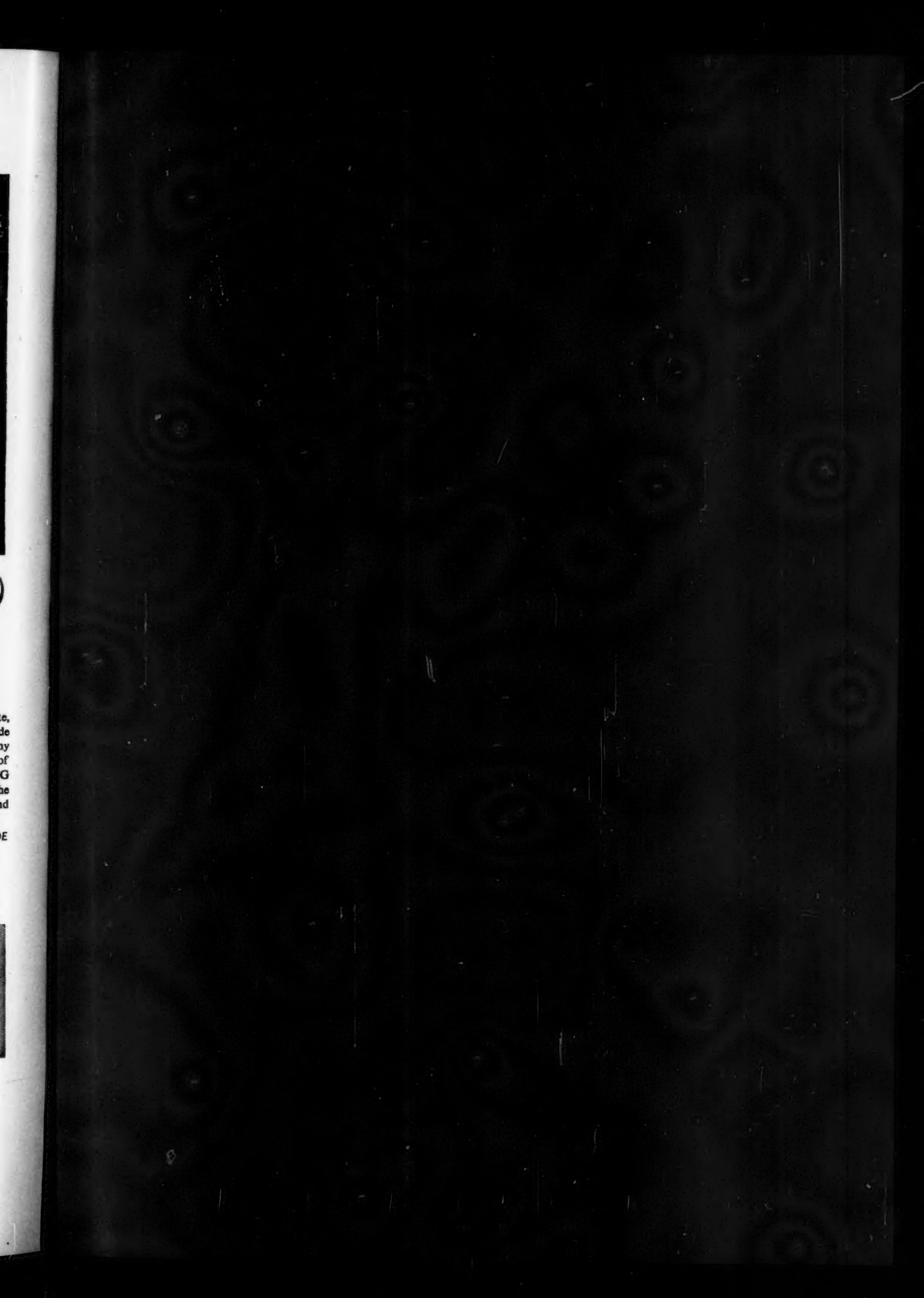

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